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**WHOLE CELL ENGINEERING
BY MUTAGENIZING A SUBSTANTIAL PORTION
OF A STARTING GENOME,
COMBINING MUTATIONS,
AND OPTIONALLY REPEATING**

signature

Mikhail Bayley
name

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CROSS-REFERENCE TO RELATED APPLICATIONS

This application ^{is a continuation-in-part} ~~claims priority~~ under Section 120 to U.S. Patent Application No. 09/594,459, filed June 14, 2000, which is a continuation-in-part of U.S. Patent Application No. 09/522,289, filed March 9, 2000, ^{PAT NO. 6,358,709} which is a continuation-in-part of U.S. Patent Application No. 09/498,557, filed February 4, 2000, which is a continuation-in-part of U.S. Patent Application No. 09/495,052, filed January 31, 2000. This application also claims priority under Section 119(e)(1) to U.S. Provisional Patent Application No. 60/156,815, filed September 29, 1999.

A - FIELD OF THE INVENTION

This invention relates to the field of cellular and whole organism engineering. Specifically, this invention relates to a cellular transformation, directed evolution, and screening method for creating novel transgenic organisms having desirable properties. Thus in one aspect, this invention relates to a method of generating a transgenic organism, such as a microbe or a plant, having a plurality of traits that are differentially activatable.

This invention also relates to the field of protein engineering. Specifically, this invention relates to a directed evolution method for preparing a polynucleotide encoding a polypeptide. More specifically, this invention relates to a method of using mutagenesis to generate a novel polynucleotide encoding a novel polypeptide, which novel polypeptide is itself an improved biological molecule &/or contributes to the generation of another improved biological molecule. More specifically still, this invention relates to a method of performing both non-stochastic polynucleotide chimerization and non-stochastic site-directed point mutagenesis.

Thus, in one aspect, this invention relates to a method of generating a progeny set of chimeric polynucleotide(s) by means that are synthetic and non-stochastic, and where the design of the progeny polynucleotide(s) is derived by analysis of a parental set of polynucleotides &/or of the polypeptides correspondingly encoded by the parental

polynucleotides. In another aspect this invention relates to a method of performing site-directed mutagenesis using means that are exhaustive, systematic, and non-stochastic.

Furthermore this invention relates to a step of selecting from among a generated set of progeny molecules a subset comprised of particularly desirable species, including by a process termed end-selection, which subset may then be screened further. This invention also relates to the step of screening a set of polynucleotides for the production of a polypeptide &/or of another expressed biological molecule having a useful property.

Novel biological molecules whose manufacture is taught by this invention include genes, gene pathways, and any molecules whose expression is affected thereby, including directly encoded polypeptides &/or any molecules affected by such polypeptides. Said novel biological molecules include those that contain a carbohydrate, a lipid, a nucleic acid, &/or a protein component, and specific but non-limiting examples of these include antibiotics, antibodies, enzymes, and steroidal and non-steroidal hormones.

In a particular non-limiting aspect, the present invention relates to enzymes, particularly to thermostable enzymes, and to their generation by directed evolution. More particularly, the present invention relates to thermostable enzymes which are stable at high temperatures and which have improved activity at lower temperatures.

B - BACKGROUND

General Overview of the Problem to Be Solved

Brief Summary: It is instantly appreciated that the process of performing a genetic manipulation on a organism to achieve a genetic alteration, whether it is on a unicellular or on a multi-cellular organism, can lead to harmful, toxic, noxious, or even lethal effects on the manipulated organism. This is particularly true when the genetic manipulation becomes sizable. From a technical point of view, this problem is seen as one of the current obstacles that hinder the creation of genetically altered organisms having a large number of transgenic traits.

On the marketing side, is instantly appreciated that the purchase price of a genetically altered organism is often dictated by, or proportional to, the number of transgenic traits that have been introduced into the organism. Consequently, a genetically altered organism having a large number of stacked transgenic traits can be quite costly to produce and purchase and economically in low demand.

On the other hand, the generation of organism having but a single genetically introduced trait can also lead to the incurrence of undesirable costs, although for other reasons. It is thus appreciated that the separate production, marketing, & storage of genetically altered organisms each having a single transgenic traits can incur costs, including inventory costs, that are undesirable. For example, the storage of such organisms may require a separate bin to be used for each trait. Furthermore, the value of an organisms having a single particular trait is often intimately tied to the marketability of that particular trait, and when that marketability diminishes, inventories of such organisms cannot be sold in other markets.

The instant invention solves these and other problems by providing a method of producing genetically altered organisms having a large number of stacked traits that are differentially activatable. Upon purchasing such a genetically altered organism (having a large number of differentially activatable stacked traits), the purchasing customer has the option of selecting and paying for particular traits among the total that can then be activated differentially. One economic advantage provided by this invention is that the storage of such genetically altered organisms is simplified since, for example, one bin could be used to store a large number of traits. Moreover, a single organism of this type can satisfy the demands for a variety of traits; consequently, such an organism can be sold in a variety of markets.

To achieve the production of genetically altered organisms having a large number of stacked traits that are differentially activatable, this invention provides - in one specific aspect - a process comprising the step of monitoring a cell or organism at holistic level. This serves as a way of collecting holistic - rather than isolated - information about a working cell or organism that is being subjected to a substantial amount of genetic manipulation. This invention further provides that this type of holistic monitoring can include the detection of all morphological, behavioral, and physical parameters.

Accordingly, the holistic monitoring provided by this invention can include the identification &/or quantification of all the genetic material contained in a working cell or organism (e.g. all nucleic acids including the entire genome, messenger RNA's, tRNA's, rRNA's, and mitochondrial nucleic acids, plasmids, phages, phagemids, viruses, as well as all episomal nucleic acids and endosymbiont nucleic acids). Furthermore this invention provides that this type of holistic monitoring can include all gene products produced by the working cell or organisms.

Furthermore, the holistic monitoring provided by this invention can include the identification &/or quantification of all molecules that are chemically at least in part protein in a working cell or organism. The holistic monitoring provided by this invention can also include the identification &/or quantification of all molecules that are chemically at least in part carbohydrate in a working cell or organism. The holistic monitoring provided by this invention can also include the identification &/or quantification of all molecules that are chemically at least in part proteoglycan in a working cell or organism. The holistic monitoring provided by this invention can also include the identification &/or quantification of all molecules that are chemically at least in part glycoprotein in a working cell or organism. The holistic monitoring provided by this invention can also include the identification &/or quantification of all molecules that are chemically at least in part nucleic acids in a working cell or organism. The holistic monitoring provided by this invention can also include the identification &/or quantification of all molecules that are chemically at least in part lipids in a working cell or organism.

In one aspect, this invention provides that the ability to differentially activate a trait from among many, such as a enzyme from among many enzymes, depends the enzyme(s) to be activated having a unique activity profile (or activity fingerprint). An enzyme's activity profile includes the reaction(s) it catalyzes and its specificity. Thus, an enzymes activity profile includes its:

- Catalyzed reaction(s)
- Reaction type
- Natural substrate(s)
- Substrate spectrum
- Product spectrum
- Inhibitor(s)
- Cofactor(s)/prostetic group(s)
- Metal compounds/salts that affect it
- Turnover number
- Specific activity
- Km value
- pH optimum
- pH range
- Temperature optimum
- Temperature range

It is also instantly appreciated that enzymes are differentially affected by exposure to varying degrees of processing (e.g. upon extraction &/or purification) and exposure (e.g.

to suboptimal storage conditions). Accordingly, enzyme differences may surface after exposure to:

- Isolation/Preparation
- Purification
- Crystallization
- Renaturation

It is instantly appreciated that differences in molecular stability can also be used advantageously to differentially activate or inactivate selected enzymes, by exposing the enzymes for an appropriate time to variations in:

- pH
- Temperature
- Oxidation
- Organic solvent(s)
- Miscellaneous storage conditions

It is thus appreciated that in order to be able to differentially activate selected traits among a plurality of stacked traits, it is desirable to introduce into a working cell or organism traits conferred by molecules (e.g. enzymes) having very unique profiles (e.g. unique enzyme fingerprints). Furthermore, it is appreciated that in order to obtain the molecules having a representation of a wide range of molecular fingerprints, it is advantageous to harvest molecules from the widest possible reaches nature's diversity. Thus, it is beneficial to harvest molecules not only from cultured mesophilic organisms, but also from extremophiles that are largely uncultured.

In another aspect, it is instantly appreciated that harvesting the full potential of nature's diversity can include both the step of discovery and the step of optimizing what is discovered. For example, the step of discovery allows one to mine biological molecules that have commercial utility. It is instantly appreciated that the ability to harvest the full richness of biodiversity, i.e. to mine biological molecules from a wide range of environmental conditions, is critical to the ability to discover novel molecules adapted to function under a wide variety of conditions, including extremes of conditions, such as may be found in a commercial application.

However, it is also instantly appreciated that only occasionally are there criteria for selection &/or survival in nature that point in the exact direction of particular commercial needs. Instead, it is often the case that a naturally occurring molecule will require a certain amount of change – from fine tuning to sweeping modification – in order to fulfill a

particular unmet commercial need. Thus, to meet certain commercial needs (e.g., a need for a molecule that is functional under a specific set of commercial processing conditions) it is sometimes advantageous to experimentally modify a naturally expressed molecule to achieve properties beyond what natural evolution has provided &/or is likely to provide in the near future.

The approach, termed directed evolution, of experimentally modifying a biological molecule towards a desirable property, can be achieved by mutagenizing one or more parental molecular templates and by identifying any desirable molecules among the progeny molecules. Currently available technologies in directed evolution include methods for achieving stochastic (i.e. random) mutagenesis and methods for achieving non-stochastic (non-random) mutagenesis. However, critical shortfalls in both types of methods are identified in the instant disclosure.

In prelude, it is noteworthy that it may be argued philosophically by some that all mutagenesis – if considered from an objective point of view – is non-stochastic; and furthermore that the entire universe is undergoing a process that – if considered from an objective point of view – is non-stochastic. Whether this is true is outside of the scope of the instant consideration. Accordingly, as used herein, the terms “randomness”, “uncertainty”, and “unpredictability” have subjective meanings, and the knowledge, particularly the predictive knowledge, of the designer of an experimental process is a determinant of whether the process is stochastic or non-stochastic.

By way of illustration, stochastic or random mutagenesis is exemplified by a situation in which a progenitor molecular template is mutated (modified or changed) to yield a set of progeny molecules having mutation(s) that are not predetermined. Thus, in an in vitro stochastic mutagenesis reaction, for example, there is not a particular predetermined product whose production is intended; rather there is an uncertainty – hence randomness – regarding the exact nature of the mutations achieved, and thus also regarding the products generated. In contrast, non-stochastic or non-random mutagenesis is exemplified by a situation in which a progenitor molecular template is mutated (modified or changed) to yield a progeny molecule having one or more predetermined mutations. It is appreciated that the presence of background products in some quantity is a reality in many reactions where molecular processing occurs, and the presence of these background products does not detract from the non-stochastic nature of a mutagenesis process having a predetermined product.

propagation of a biological molecule is susceptible to any global detrimental effects – whether caused by itself or not – on its ecosystem. These and other characteristics greatly limit the types of mutations that can be propagated in nature.

On the other hand, directed (or experimental) evolution – particularly as provided herein – can be performed much more rapidly and can be directed in a more streamlined manner at evolving a predetermined molecular property that is commercially desirable where nature does not provide one &/or is not likely to provide. Moreover, the directed evolution invention provided herein can provide more wide-ranging possibilities in the types of steps that can be used in mutagenesis and selection processes. Accordingly, using templates harvested from nature, the instant directed evolution invention provides more wide-ranging possibilities in the types of progeny molecules that can be generated and in the speed at which they can be generated than often nature itself might be expected to in the same length of time.

In a particular exemplification, the instantly disclosed directed evolution methods can be applied iteratively to produce a lineage of progeny molecules (e.g. comprising successive sets of progeny molecules) that would not likely be propagated (i.e., generated &/or selected for) in nature, but that could lead to the generation of a desirable downstream mutagenesis product that is not achievable by natural evolution.

Previous Directed Evolution Methods Are Suboptimal:

Mutagenesis has been attempted in the past on many occasions, but by methods that are inadequate for the purpose of this invention. For example, previously described non-stochastic methods have been serviceable in the generation of only very small sets of progeny molecules (comprised often of merely a solitary progeny molecule). By way of illustration, a chimeric gene has been made by joining 2 polynucleotide fragments using compatible sticky ends generated by restriction enzyme(s), where each fragment is derived from a separate progenitor (or parental) molecule. Another example might be the mutagenesis of a single codon position (i.e. to achieve a codon substitution, addition, or deletion) in a parental polynucleotide to generate a single progeny polynucleotide encoding for a single site-mutagenized polypeptide.

Previous non-stochastic approaches have only been serviceable in the generation of but one to a few mutations per method application. Thus, these previously described non-stochastic methods thus fail to address one of the central goals of this invention, namely the exhaustive and non-stochastic chimerization of nucleic acids. Accordingly previous non-

stochastic methods leave untapped the vast majority of the possible point mutations, chimerizations, and combinations thereof, which may lead to the generation of highly desirable progeny molecules.

In contrast, stochastic methods have been used to achieve larger numbers of point mutations and/or chimerizations than non-stochastic methods; for this reason, stochastic methods have comprised the predominant approach for generating a set of progeny molecules that can be subjected to screening, and amongst which a desirable molecular species might hopefully be found. However, a major drawback of these approaches is that – because of their stochastic nature – there is a randomness to the exact components in each set of progeny molecules that is produced. Accordingly, the experimentalist typically has little or no idea what exact progeny molecular species are represented in a particular reaction vessel prior to their generation. Thus, when a stochastic procedure is repeated (e.g. in a continuation of a search for a desirable progeny molecule), the re-generation and re-screening of previously discarded undesirable molecular species becomes a labor-intensive obstruction to progress, causing a circuitous – if not circular – path to be taken. The drawbacks of such a highly suboptimal path can be addressed by subjecting a stochastically generated set of progeny molecules to a labor-incurring process, such as sequencing, in order to identify their molecular structures, but even this is an incomplete remedy.

Moreover, current stochastic approaches are highly unsuitable for comprehensively or exhaustively generating all the molecular species within a particular grouping of mutations, for attributing functionality to specific structural groups in a template molecule (e.g. a specific single amino acid position or a sequence comprised of two or more amino acids positions), and for categorizing and comparing specific grouping of mutations. Accordingly, current stochastic approaches do not inherently enable the systematic elimination of unwanted mutagenesis results, and are, in sum, burdened by too many inherently shortcomings to be optimal for directed evolution.

In a non-limiting aspect, the instant invention addresses these problems by providing non-stochastic means for comprehensively and exhaustively generating all possible point mutations in a parental template. In another non-limiting aspect, the instant invention further provides means for exhaustively generating all possible chimerizations within a group of chimerizations. Thus, the aforementioned problems are solved by the instant invention.

Specific shortfalls in the technological landscape addressed by this invention include:

1) Site-directed mutagenesis technologies, such as sloppy or low-fidelity PCR, are ineffective for systematically achieving at each position (site) along a polypeptide sequence the full (saturated) range of possible mutations (i.e. all possible amino acid substitutions).

2) There is no relatively easy systematic means for rapidly analyzing the large amount of information that can be contained in a molecular sequence and in the potentially colossal number or progeny molecules that could be conceivably obtained by the directed evolution of one or more molecular templates.

3) There is no relatively easy systematic means for providing comprehensive empirical information relating structure to function for molecular positions.

4) There is no easy systematic means for incorporating internal controls, such as positive controls, for key steps in certain mutagenesis (e.g. chimerization) procedures.

5) There is no easy systematic means to select for a specific group of progeny molecules, such as full-length chimeras, from among smaller partial sequences.

An exceedingly large number of possibilities exist for the purposeful and random combination of amino acids within a protein to produce useful hybrid proteins and their corresponding biological molecules encoding for these hybrid proteins, i.e., DNA, RNA. Accordingly, there is a need to produce and screen a wide variety of such hybrid proteins for a desirable utility, particularly widely varying random proteins.

The complexity of an active sequence of a biological macromolecule (e.g., polynucleotides, polypeptides, and molecules that are comprised of both polynucleotide and polypeptide sequences) has been called its information content ("IC"), which has been defined as the resistance of the active protein to amino acid sequence variation (calculated from the minimum number of invariable amino acids (bits) required to describe a family of related sequences with the same function). Proteins that are more sensitive to random mutagenesis have a high information content.

Molecular biology developments, such as molecular libraries, have allowed the identification of quite a large number of variable bases, and even provide ways to select functional sequences from random libraries. In such libraries, most residues can be varied (although typically not all at the same time) depending on compensating changes in the context. Thus, while a 100 amino acid protein can contain only 2,000 different mutations, 20^{100} sequence combinations are possible.

Error-prone PCR and oligonucleotide-directed mutagenesis are thus useful for single cycles of sequence fine-tuning, but rapidly become too limiting when they are applied for multiple cycles.

Another limitation of error-prone PCR is that the rate of down-mutations grows with the information content of the sequence. As the information content, library size, and mutagenesis rate increase, the balance of down-mutations to up-mutations will statistically prevent the selection of further improvements (statistical ceiling).

In cassette mutagenesis, a sequence block of a single template is typically replaced by a (partially) randomized sequence. Therefore, the maximum information content that can be obtained is statistically limited by the number of random sequences (i.e., library size). This eliminates other sequence families which are not currently best, but which may have greater long term potential.

Also, mutagenesis with synthetic oligonucleotides requires sequencing of individual clones after each selection round. Thus, such an approach is tedious and impractical for many rounds of mutagenesis.

Thus, error-prone PCR and cassette mutagenesis are best suited, and have been widely used, for fine-tuning areas of comparatively low information content. One apparent exception is the selection of an RNA ligase ribozyme from a random library using many rounds of amplification by error-prone PCR and selection.

In nature, the evolution of most organisms occurs by natural selection and sexual reproduction. Sexual reproduction ensures mixing and combining of the genes in the offspring of the selected individuals. During meiosis, homologous chromosomes from the parents line up with one another and cross-over part way along their length, thus randomly swapping genetic material. Such swapping or shuffling of the DNA allows organisms to evolve more rapidly.

In recombination, because the inserted sequences were of proven utility in a homologous environment, the inserted sequences are likely to still have substantial information content once they are inserted into the new sequence.

Theoretically there are 2,000 different single mutants of a 100 amino acid protein. However, a protein of 100 amino acids has 20^{100} possible sequence combinations, a number which is too large to exhaustively explore by conventional methods. It would be

advantageous to develop a system which would allow generation and screening of all of these possible combination mutations.

Some workers in the art have utilized an *in vivo* site specific recombination system to generate hybrids of combine light chain antibody genes with heavy chain antibody genes for expression in a phage system. However, their system relies on specific sites of recombination and is limited accordingly. Simultaneous mutagenesis of antibody CDR regions in single chain antibodies (scFv) by overlapping extension and PCR have been reported.

Others have described a method for generating a large population of multiple hybrids using random *in vivo* recombination. This method requires the recombination of two different libraries of plasmids, each library having a different selectable marker. The method is limited to a finite number of recombinations equal to the number of selectable markers existing, and produces a concomitant linear increase in the number of marker genes linked to the selected sequence(s).

In vivo recombination between two homologous, but truncated, insect-toxin genes on a plasmid has been reported as a method of producing a hybrid gene. The *in vivo* recombination of substantially mismatched DNA sequences in a host cell having defective mismatch repair enzymes, resulting in hybrid molecule formation has been reported.

C - SUMMARY OF THE INVENTION

This invention relates generally to the field of cellular and whole organism engineering. Specifically, this invention relates to a cellular transformation, directed evolution, and screening method for creating novel transgenic organisms having desirable properties. Thus in one aspect, this invention relates to a method of generating a transgenic organism, such as a microbe or a plant, having a plurality of traits that are differentially activatable.

In one embodiment, this invention is directed to a method of producing an improved organism having a desirable trait to by: a) obtaining an initial population of organisms, b) generating a set of mutagenized organisms, such that when all the genetic mutations in the set of mutagenized organisms are taken as a whole, there is represented a set of substantial genetic mutations, and c) detecting the presence of said improved organism. This invention provides that any of steps a), b), and c) can be further repeated

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population of organisms", which refers simply to a population of organisms with which one is working, and which is comprised of at least one organism. An **"initial population of organisms"** which can be a clonal population or otherwise.

Accordingly, in step 1) an "initial population of organisms" may be a population of multicellular organisms or of unicellular organisms or of both. An "initial population of organisms" may be comprised of unicellular organisms or multicellular organisms or both. An "initial population of organisms" may be comprised of prokaryotic organisms or eukaryotic organisms or both. This invention provides that an "initial population of organisms" is comprised of at least one organism, and preferred embodiments include at least that .

Non-limiting particularly preferred examples of kinds of “**organisms**” also include Archaea (archaeobacteria) and Bacteria (eubacteria). Non-limiting examples of Archaea (archaeobacteria) include Crenarchaeota, Euryarchaeota, and Korarchaeota. Non-limiting examples Bacteria (eubacteria) include Aquificales, CFB/Green sulfur bacteria group, Chlamydiales/Verrucomicrobia group, Chrysiogenes group, Coprothermobacter group, Cyanobacteria & chloroplasts, Cytophaga/Flexibacter /Bacteriodes group, Dictyoglomus group, Fibrobacter/Acidobacteria group, Firmicutes, Flexistipes group, Fusobacteria, Green non-sulfur bacteria, Nitrospira group, Planctomycetales, Proteobacteria, Spirochaetales, Synergistes group, Thermodesulfobacterium group, Thermotogales, Thermus/Deinococcus group. As non-limiting examples, particularly preferred kinds of organisms include Aquifex, Aspergillus, Bacillus, Clostridium, E. coli, Lactobacillus, Mycobacterium, Pseudomonas, Streptomyces, and Thermotoga. As additional non-limiting examples, particularly preferred organisms include cultivated organisms such as CHO, VERO, BHK, HeLa, COS, MDCK, Jurkat, HEK-293, and WI38. Particularly preferred non-limiting examples of organisms further include host organisms that are

serviceable for the expression of recombinant molecules. Organisms further include primary cultures (e.g. cells from harvested mammalian tissues), immortalized cells, all cultivated and culturable cells and multicellular organisms, and all uncultivated and unculturable cells and multicellular organisms.

In a preferred embodiment, knowledge of genomic information is useful for performing the claimed methods; thus, this invention provides the following as preferred but non-limiting examples of organisms that are particularly serviceable for this invention, because there is a significant amount of - if not complete - genomic sequence information (in terms of primary sequence &/or annotation) for these organisms: Human, Insect (e.g. *Drosophila melanogaster*), Higher plants (e.g. *Arabidopsis thaliana*), Protozoan (e.g. *Plasmodium falciparum*), Nematode (e.g. *Caenorhabditis elegans*), Fungi (e.g. *Saccharomyces cerevisiae*), Proteobacteria gamma subdivision (e.g. *Escherichia coli* K-12, *Haemophilus influenzae* Rd, *Xylella fastidiosa* 9a5c, *Vibrio cholerae* El Tor N16961, *Pseudomonas aeruginosa* PA01, *Buchnera* sp. APS), Proteobacteria beta subdivision (e.g. *Neisseria meningitidis* MC58 (serogroup B), *Neisseria meningitidis* Z2491 (serogroup A)), Proteobacteria other subdivisions (e.g. *Helicobacter pylori* 26695, *Helicobacter pylori* J99, *Campylobacter jejuni* NCTC11168, *Rickettsia prowazekii*), Gram-positive bacteria (e.g. *Bacillus subtilis*, *Mycoplasma genitalium*, *Mycoplasma pneumoniae*, *Ureaplasma urealyticum*, *Mycobacterium tuberculosis* H37Rv), Chlamydia (e.g. *Chlamydia trachomatis* serovar D, *Chlamydia muridarum* (*Chlamydia trachomatis* MoPn), *Chlamydia pneumoniae* CWL029, *Chlamydia pneumoniae* AR39, *Chlamydia pneumoniae* J138), Spirochete (e.g. *Borrelia burgdorferi* B31, *Treponema pallidum*), Cyanobacteria (e.g. *Synechocystis* sp. PCC6803), Radioresistant bacteria (e.g. *Deinococcus radiodurans* R1), Hyperthermophilic bacteria (e.g. *Aquifex aeolicus* VF5, *Thermotoga maritima* MSB8), and Archaea (e.g. *Methanococcus jannaschii*, *Methanobacterium thermoautotrophicum* deltaH, *Archaeoglobus fulgidus*, *Pyrococcus horikoshii* OT3, *Pyrococcus abyssi*, *Aeropyrum pernix* K1).

Non-limiting particularly preferred examples of kinds of plant "organisms" include those listed in Table 1.

Table 1. Non-limiting examples of plant organisms and sources of transgenic molecules
(e.g. nucleic acids & nucleic acid products)

1. Alfalfa	39. Pepper
2. Amelanchier laevis	40. Persimmon
3. Apple	41. Petunia
4. Arab. thaliana	42. Pine
5. Arabidopsis	43. Pineapple
6. Aspergillus flavus	44. Pink bollworm
7. Barley	45. Plum
8. Beet	46. Poplar
9. Belladonna	47. Potato
10. Brassica oleracea	48. Pseudomonas
11. Carrot	49. Pseudomonas putida
12. Chrysanthemum	50. Pseudomonas syringae
13. Cichorium intybus	51. Rapeseed
14. Clavibacter	52. Rhizobium
15. Clavibacter xyli	53. Rhizobium etli
16. Coffee	54. Rhizobium fredii
17. Corn	55. Rhizobium leguminosarum
18. Cotton	56. Rhizobium meliloti
19. Cranberry	57. Rice
20. Creeping bentgrass	58. Rubus idaeus
21. Cryphonectria parasitica	59. Spruce
22. Eggplant	60. Soybean
23. Festuca arundinacea	61. Squash
24. Fusarium graminearum	62. Squash-cucumber
25. Fusarium moniliforme	63. Squash-cucurbita texana
26. Fusarium sporotrichioides	64. Strawberry
27. Gladiolus	65. Sugarcane
28. Grape	66. Sunflower
29. Heterorhabditis bacteriophora	67. Sweet potato
30. Kentucky bluegrass	68. Sweetgum
31. Lettuce	69. TMV
32. Melon	70. Tobacco
33. Oat	71. Tomato
34. Onion	72. Walnut
35. Papaya	73. Watermelon
36. Pea	74. Wheat
37. Peanut	75. Xanthomonas
38. Pelargonium	76. Xanthomonas campestris

As used herein, the meaning of “generating a set of mutagenized organisms having genetic mutations” includes the steps of substituting, deleting, as well as introducing a nucleotide sequence into organism; and this invention provides a nucleotide sequence that serviceable for this purpose may be a single-stranded or double-stranded and the fact that its length may be from one nucleotide up to 10,000,000,000 nucleotides in length including specifically every integer value in between.

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expression pattern (e.g. throughout a period of time) of at least about 1% to about 100% of the gene products &/or phenotypes &/or traits of an organism (or type of organism), including specifically every integer value in between.

In yet another aspect, as used herein, a **"set of substantial genetic mutations"** is preferably an introduction or deletion of at least about 15 to 150,000 genes promoters or other nucleotide sequences (where each sequence is from 1 base to 10,000,000 bases), including specifically every integer value in between. For example, one can introduce a library of at least about 15 to 150,000 nucleotides (genes or promoters) produced by "site-saturation mutagenesis" &/or by "ligation reassembly" (including any specific aspect thereof provided herein) into an **"initial population of organisms"**.

It is provided that wherever the manipulation of a plurality of **"genes"** is mentioned herein, gene pathways (e.g. that ultimately lead to the production of small molecules) are also included. It is appreciated herein that knocking-out, altering expression level, and altering expression pattern can be achieved, by non-limiting exemplification, by mutagenizing a nucleotide sequence corresponding gene as well as a corresponding promoter that affects the expression of the gene.

As used herein, a **"mutagenized organism"** includes any organism that has been altered by a genetic mutation.

A **"genetic mutation"** can be, by way of non-limiting and non-mutually exclusive exemplification, and change in the nucleotide sequence (DNA or RNA) with respect to genomic, extra-genomic, episomal, mitochondrial, and any nucleotide sequence associated with (e.g. contained within or considered part of) an organism..

According to this invention, detecting the manifestation of a **"genetic mutation"** means **"detecting the manifestation of a detectable parameter"**, including but not limited to a change in the genomic sequence. Accordingly, this invention provides that a step of sequencing (&/or annotating) of and organism's genomic DNA is necessary for some methods of this invention, and exemplary but non-limiting aspects of this sequencing (&/or annotating) step are provided herein.

A detectable “**trait**”, as used herein, is any detectable parameter associated with the organism. Accordingly, such a detectable “parameter” includes, by way of non-limiting exemplification, any detectable “nucleotide knock-in”, any detectable “nucleotide knock-outs”, any detectable “phenotype”, and any detectable “genotype”. By way of further illustration, a “**trait**” includes any substance produced or not produced by the organism. Accordingly, a “**trait**” includes viability or non-viability, behavior, growth rate, size, morphology. “**Trait**” includes increased (or alternatively decreased) expression of a gene product or gene pathway product. “**Trait**” also includes small molecule production (including vitamins, antibiotics), herbicide resistance, drought resistance, pest resistance, production of any recombinant biomolecule (ie.g. vaccines, enzymes, protein therapeutics, chiral enzymes). Additional examples of serviceable traits for this invention are shown in Table 2.

TABLE 2 – Non-limiting examples of serviceable genes, gene products, phenotypes, or traits according to the methods of this invention (e.g. knockouts, knockins, increased or decreased expression level, increased or decreased expression pattern)

Table 2 - Part 1. Non-limiting examples of genes or gene products

1. 17 kDa protein	53. Cecropin
2. 3-hydroxy-3-methylglutaryl CoenzymeA reductase	54. Cecropin B
3. 4-Coumarate:CoA ligase knockout	55. Cellulose binding protein
4. 60 kDa protein	56. Chalcone synthase knockout
5. Ac transposable element	57. Chitinase
6. ACC deaminase	58. Chitobiosidase
7. ACC oxidase knockout	59. Chloramphenicol acetyltransferase
8. ACC synthase	60. Cholera toxin B
9. ACC synthase knockout	61. Choline oxidase
10. Acetohydroxyacid synthase variant	62. Cinnamate 4-hydroxylase
11. Acetolactate synthase	63. Cinnamate 4-hydroxylase knockout
12. Acetyl CoA carboxylase	64. Coat protein
13. ACP acyl-ACP thioesterase	65. Coat protein knockout
14. ACP thioesterase	66. Conglycinin
15. Acyl CoA reductase	67. CryIA
16. Acyl-ACP knockout	68. CryIAb
17. Acyl-ACP desaturase	69. CryIAc
18. Acyl-ACP desaturase knockout	70. CryIB
19. Acyl-ACP thioesterase	71. CryIIA
20. ADP glucose pyrophosphorylase	72. CryIIIA
21. ADP glucose pyrophosphorylase knockout	73. CryVIA
22. Agglutinin	74. Cyclin dependent kinase
23. Aleurone I	75. Cyclodextrin glycosyltransferase
24. Alpha hordothinonin	76. Cylindrical inclusion protein
25. Alpha-amylase	77. Cystathionine synthase
26. Alpha-hemoglobin	78. Delta-12 desaturase

27. Aminoglycoside 3'-adenylyltransferase	79. Delta-12 desaturase knockout
28. Amylase	80. Delta-12 saturase
29. Anionic peroxidase	81. Delta-12 saturase knockout
30. Antibody	82. Delta-15 desaturase
31. Antifungal protein	83. Delta-15 desaturase knockout
32. Antithrombin	84. Delta-9 desaturase
33. Antitrypsin	85. Delta-9 desaturase knockout
34. Antiviral protein	86. Deoxyhypusine synthase (DHS)
35. Aspartokinase	87. Deoxyhypusine synthase knockout
36. Attacin E	88. Diacylglycerol acetyl transferase
37. B1 regulatory gene	89. Dihydrodipicolinate synthase
38. B-1,3-glucanase knockout	90. Dihydrofolate reductase
39. B-1,4-endoglucanase knockout	91. Diptheria toxin A
40. Bacteropsin	92. Disease resistance response gene 49
41. Barnase	93. Double stranded ribonuclease
42. Barstar	94. Ds transposable element
43. Beta-hemoglobin	95. Elongase
44. B-glucuronidase	96. EPSPS
45. C1 knockout	97. Ethylene forming enzyme knockout
46. C1 regulatory gene	98. Ethylene receptor protein
47. C2 knockout	99. Ethylene receptor protein knockout
48. C3 knockout	100. Fatty acid elongase
49. Caffeate O-methyltransferase	101. Fluorescent protein
50. Caffeate O-methyltransferase knockout	102. G glycoprotein
51. Caffeoyl CoA O-methyltransferase knockout	103. Galactanase
52. Casein	104. Galanthus nivalis agglutinin

Table 2 – Part 1.(continued) Non-limiting examples of transgenic genes & gene knockouts

105. Genome-linked protein	157. Omega 3 desaturase knockout
106. Glucanase	158. Omega 6 desaturase
107. Glucanase knockout	159. Omega 6 desaturase knockout
108. Glucose oxidase	160. O-methyltransferase
109. Glutamate dehydrogenase	161. Osmotin
110. Glutamine binding protein	162. Oxalate oxidase
111. Glutamine synthetase	163. Par locus
112. Glutenin	164. Pathogenesis protein 1a
113. Glycerol-3-phosphate acetyl transferase	165. Pectate lyase
114. Glyphosate oxidoreductase	166. Pectin esterase
115. Glyphosate oxidoreductase	167. Pectin esterase knockout
116. Green fluorescent protein	168. Pectin methylesterase
117. Helper component	169. Pectin methylesterase knockout
118. Hemicellulase	170. Pentenlypyrophosphate isomerase
119. Hup locus	171. Phosphinothricin
120. Hygromycin phosphotransferase	172. Phosphinothricin acetyl transferase
121. Hyoscamine 6B-hydroxylase	173. Phytochrome A
122. IAA monooxygenase	174. Phytoene synthase
123. Invertase	175. Phleomycin binding protein
124. Invertase knockout	176. Polygalacturonase
125. Isopentenyl transferase	177. Polygalacturonase knockout
126. Ketoacyl-ACP synthase	178. Polygalacturonase inhibitor protein
127. Ketoacyl-ACP synthase knockout	179. Prf regulatory gene
128. Larval serum protein	180. Prosystemin
129. Leafy homeotic regulatory gene	181. Protease
130. Lectin	182. Protein A
131. Lignin peroxidase	183. Protein kinase
132. Luciferase	184. Proteinase inhibitor 1
133. Lysine-2 gene	185. Pti5 transcription factor
134. Lysophosphatidic acid acetyl transferase	186. R regulatory gene
135. Lysozyme	187. Receptor kinase
136. Mabinlin	188. Recombinase

137. Male sterility protein	189. Reductase
138. Metallothionein	190. Replicase
139. Modified ethylene receptor protein	191. Resveratrol synthase
140. Modified ethylene receptor protein knockout	192. Ribonuclease
141. Monooxygenase	193. rolc
142. Movement protein	194. Rol hormone gene
143. Movement protein nonfunctional	195. S-adenosylmethione decarboxylase
144. N gene for TMV resistance	196. S-adenosylmethione hydrolase
145. N-acetyl glucosidase	197. S-adenosylmethionine transferase
146. Nitrilase	198. Salicylate hydroxylase
147. Nopaline synthase	199. Satellite RNA
148. Notch	200. Seed storage protein
149. NptII	201. Serine-threonine protein kinase
150. Nuclear inclusion protein a	202. Serum albumin
151. Nuclear inclusion protein b	203. Shrunk 2
152. Nucleocapsid	204. Sorbitol dehydrogenase
153. Nucleoprotein	205. Sorbitol synthase
154. O-acyl transferase	206. Stilbene synthase
155. Oleoyl-ACP thioesterase	207. Storage protein
156. Omega 3 desaturase	208. Sucrose phosphate synthase

Table 2 – Part 1.(continued) Non-limiting examples of transgenic genes & gene knockouts

209. Systemic acquired resistance gene 8.2	219. Trichosanthin
210. Tetracycline binding protein	220. Trifolitoxin
211. Thioesterase (x2)	221. Trypsin inhibitor
212. Thiolase	222. T-URF13 mitochondrial
213. TobRB7	223. UDP glucose glucosyltransferase
214. Transcriptional activator	224. Violaxanthin de-epoxidase
215. Transposon Tn5	225. Violaxanthin de-epoxidase knockout
216. Trehalase	226. Wheat germ agglutinin
217. Trehalase knockout	227. Xanthosine-N7-methyltransferase knockout
218. Trichodiene synthase	228. Zein storage protein

Table 2 – Part 2. Non-limiting examples of input traits/phenotypes

1. 2,4-D tolerant	52. Flowering time altered
2. Alternaria resistant	53. Frog-eye leaf spot resistant
3. Altered amino acid composition	54. Fruit ripening altered
4. Alternaria solani resistant	55. Fruit ripening delayed
5. Ammonium assimilation increased	56. Fruit rot resistant
6. AMV resistant	57. Fruit solids increased
7. Aphid resistant	58. Fruit sweetness increased
8. Apple scab resistant	59. Fungal post-harvest resistant
9. Aspergillus resistant	60. Fungal resistant
10. B-1,4-endoglucanase	61. Fungal resistant general
11. Bacterial leaf blight resistant	62. Fusarium resistant
12. Bacterial speck resistant	63. Glyphosate tolerant
13. BCTV resistant	64. Growth rate altered
14. Blackspot bruise resistant	65. Growth rate reduced
15. BLRV resistant	66. Heat stable glucanase produced
16. BNYVV Resistant	67. Hordothionin produced
17. Botrytis cinerea resistant	68. Imidazolinone tolerant
18. Botrytis resistant	69. Insect resistant general
19. BPMV resistant	70. Kanamycin resistant
20. Bromoxynil tolerant	71. Lepidopteran resistant
21. BYDV resistant	72. Lesser cornstalk borer resistant
22. BYMV resistant	73. LMV resistant
23. Carbohydrate metabolism altered	74. Loss of systemic resistance
24. Cell wall altered	75. Male sterile

25. Chlorsulfuron tolerant	76. Marssonina resistant
26. Clavibacter resistant	77. MCDV resistant
27. CLRV resistant	78. MCMV resistant
28. CMV resistant	79. MDMV resistant
29. Cold tolerant	80. MDMV-B resistant
30. Coleopteran resistant	81. Mealybug wilt virus resistant
31. Colletotrichum resistant	82. Melamtsora resistant
32. Colorado potato beetle resistant	83. Melodgyne resistant
33. Constitutive expression of glutamine synthetase	84. Methotrexate resistant
34. Corynebacterium sepedonicum resistant	85. Mexican Rice Borer resistant
35. Cottonwood leaf beetle resistant	86. Nucleocapsid protein produced
36. Crown gall resistant	87. Oblique banded leafroller resistant
37. Crown rot resistant	88. PEMV resistant
38. Cucumovirus resistant	89. PeSV resistant
39. Cutting rootability increased	90. Phoma resistant
40. Downy mildew resistant	91. Phosphinothricin tolerant
41. Drought tolerant	92. Phratora leaf beetle resistant
42. Erwinia carotovora resistant	93. Phytophthora resistant
43. Ethylene production reduced	94. PLRV resistant
44. European Corn Borer resistant	95. Polyamine metabolism altered
45. Female sterile	96. Potyvirus resistant
46. Fenthion susceptible	97. Powdery mildew resistant
47. Fertility altered	98. PPV resistant
48. Fire blight resistant	99. Pratylenchus vulnus resistant
49. Flower and fruit abscission reduced	100. Proteinase inhibitors level constitutive
50. Flower and fruit set altered	101. PRSV resistant
51. Flowering altered	102. PRV resistant

Table 2 – Part 2.(continued)Non-limiting examples of transgenic input traits/phenotypes

103. PSbMV resistant	128. Streptomyces scabies resistant
104. Pseudomonas syringae resistant	129. Sulfonylurea tolerant
105. PStV resistant	130. Tetracycline binding protein produced
106. PVX resistant	131. TEV resistant
107. PVY resistant	132. Thelaviopsis resistant
108. RBDV resistant	133. TMV resistant
109. Rhizoctonia resistant	134. Tobamovirus resistant
110. Rhizoctonia solani resistant	135. ToMoV resistant
111. Ring rot resistance	136. ToMV resistant
112. Root-knot nematode resistant	137. Transposon activator
113. SbMV resistant	138. Transposon inserted
114. Sclerotinia resistant	139. TRV resistant
115. SCMV resistant	140. TSWV resistant
116. SCYLV resistant	141. TVMV resistant
117. Secondary metabolite increased	142. TYLCV resistant
118. Seed set reduced	143. Tyrosine level increased
119. Selectable marker	144. Venturia resistant
120. Senescence altered	145. Verticillium dahliae resistant
121. Septoria resistant	146. Verticillium resistant
122. Shorter stems	147. Visual marker
123. Soft rot fungal resistant	148. WMV2 resistant
124. Soft rot resistant	149. WSMV resistant
125. SqMV resistant	150. Yield increased
126. SrMV resistant	151. ZYMV resistant
127. Storage protein altered	

Table 2 – Part 3. Non-limiting examples of output traits/phenotypes

1. ACC oxidase level decreased	36. Oil profile altered
2. Altered lignin biosynthesis	37. Pectin esterase level reduced
3. B-1,4-endoglucanase	38. Pharmaceutical proteins produced

4. Botrytis resistant	39. Phosphinothricin tolerant
5. Carbohydrate metabolism altered	40. Phytoene synthase activity increased
6. Carotenoid content altered	41. Pigment metabolism altered
7. Cell wall altered	42. Polygalacturonase level reduced
8. CMV resistant	43. Processing characteristics altered
9. Coleopteran resistant	44. Prolonged shelf life
10. Dry matter content increased	45. Protein altered
11. Ethylene production reduced	46. Protein quality altered
12. Ethylene synthesis reduced	47. PRSV resistant
13. Fatty acid metabolism altered	48. Root-knot nematode resistant
14. Fire blight resistant	49. Sclerotinia resistant
15. Flower and fruit abscission reduced	50. Seed composition altered
16. Flower and fruit set altered	51. Seed methionine storage increased
17. Flowering time altered	52. Seed set reduced
18. Fruit firmness increased	53. Seed storage protein
19. Fruit pectin esterase levels decreased	54. Senescence altered (e.g. Shelf life increased)
20. Fruit ripening altered	55. Shorter stems
21. Fruit ripening delayed	56. Solids increased
22. Fruit solids increased	57. SqMV resistant
23. Fruit sugar profile altered	58. Starch level increased
24. Fruit sweetness increased	59. Starch metabolism altered
25. Glucuronidase expressing	60. Starch reduced
26. Heat stable glucanase produced	61. Sterols increased
27. Heavy metals sequestered	62. Storage protein altered
28. Hordothionin produced	63. Sugar alcohol levels increased
29. Improved fruit quality	64. Tetracycline binding protein produced
30. Industrial enzyme produced	65. Tyrosine level increased
31. Lepidopteran resistant	66. Verticillium resistant
32. Lysine level increased	67. Visual marker
33. Mealybug wilt virus resistant	68. WMV2 resistant
34. Methionine level increased	69. Yield increased
35. Nucleocapsid protein produced	70. ZYMV resistant

Table 2 – Part 4. Non-limiting examples of traits/phenotypes with agronomic properties

1. ACC oxidase level decreased	53. Industrial enzyme produced
2. Altered amino acid composition	54. Lignin levels decreased
3. Altered lignin biosynthesis	55. Lipase expressed in seeds
4. Altered maturing	56. Lysine level increased
5. Altered plant development	57. Male sterile
6. Aluminum tolerant	58. Male sterile reversible
7. Ammonium assimilation increased	59. Methionine level increased
8. Anthocyanin produced in seed	60. Modified growth characteristics
9. B-1,4-endoglucanase	61. Mycotoxin degradation
10. Calmodulin level altered	62. Nitrogen metabolism altered
11. Carbohydrate metabolism altered	63. Nucleocapsid protein produced
12. Carotenoid content altered	64. Oil profile altered
13. Cell wall altered	65. Oil quality altered
14. Cold tolerant	66. Oxidative stress tolerant
15. Constitutive expression of glutamine synthetase	67. Pectin esterase level reduced
16. Cutting root ability increased	68. Pharmaceutical proteins produced
17. Development altered	69. Photosynthesis enhanced
18. Drought tolerant	70. Phytoene synthase activity increased
19. Dry matter content increased	71. Pigment metabolism altered
20. Environmental stress reduced	72. Polyamine metabolism altered
21. Ethylene metabolism altered	73. Polygalacturonase level reduced
22. Ethylene production reduced	74. Pratylenchus vulnus resistant
23. Ethylene synthesis reduced	75. Processing characteristics altered
24. Fatty acid metabolism altered	76. Prolonged shelf life
25. Female sterile	77. Protein altered
26. Fenthion susceptible	78. Protein lysine level increased

27. Fertility altered	79. Protein quality altered
28. Fiber quality altered	80. Proteinase inhibitors level constitutive
29. Flower and fruit abscission reduced	81. Salt tolerance increased
30. Flower and fruit set altered	82. Seed composition altered
31. Flowering altered	83. Seed methionine storage increased
32. Flower color altered	84. Seed set reduced
33. Flowering time altered	85. Selectable marker
34. Fruit firmness increased	86. Senescence altered
35. Fruit pectin esterase and levels decreased	87. Shorter stems
36. Fruit polygalacturonase level decreased	88. Solids increased
37. Fruit ripening altered	89. Starch level increased
38. Fruit ripening delayed	90. Starch metabolism altered
39. Fruit solids increased	91. Starch reduced
40. Fruit sugar profile altered	92. Sterols increased
41. Fruit sweetness increased	93. Storage protein altered
42. Glucuronidase expressing	94. Stress tolerant
43. Growth rate altered	95. Sugar alcohol levels increased
44. Growth rate increased	96. Tetracycline binding protein produced
45. Growth rate reduced	97. Thermostable protein produced
46. Heat stable glucanase produced	98. Transposon activator
47. Heat tolerant	99. Transposon inserted
48. Heavy metals sequestered	100. Tyrosine level increased
49. Hordothionin produced	101. Visual marker
50. Improved fruit quality	102. Vivipary increased
51. Increased phosphorus	103. Yield increased
52. Increased stalk strength	

Table 2 – Part 5. Non-limiting examples of traits/phenotypes with product quality properties

1. 2,4-D tolerant	45. Melanin produced in cotton fibers
2. ACC oxidase level decreased	46. Metabolism altered
3. Altered amino acid composition	47. Methionine level increased
4. Altered lignin biosynthesis	48. Mycotoxin degradation
5. Anthocyanin produced in seed	49. Mycotoxin production inhibited
6. Antioxidant enzyme increased	50. Nicotine levels reduced
7. Auxin metabolism and increased tuber solids	51. Nitrogen metabolism altered
8. B-1,4-endoglucanase	52. Novel protein produced
9. Blackspot bruise resistant	53. Nutritional quality altered
10. Brown spot resistant	54. Oil profile altered
11. Bruising reduced	55. Oil quality altered
12. Caffeine levels reduced	56. Pectin esterase level reduced
13. Carbohydrate metabolism altered	57. Photosynthesis enhanced
14. Carotenoid content altered	58. Phytoene synthase activity increased
15. Cell wall altered	59. Pigment metabolism altered
16. Cold tolerant	60. Polyamine metabolism altered
17. Delayed softening	61. Polygalacturonase level reduced
18. Disulfides reduced in endosperm	62. Processing characteristics altered
19. Dry matter content increased	63. Prolonged shelf life
20. Ear mold resistant	64. Protein altered
21. Ethylene production reduced	65. Protein lysine level increased
22. Ethylene synthesis reduced	66. Protein quality altered
23. Extended flower life	67. Proteinase inhibitors level constitutive
24. Fatty acid metabolism altered	68. Rust resistant
25. Fiber quality altered	69. Seed composition altered
26. Fiber strength altered	70. Seed methionine storage increased
27. Flavor enhancer	71. Seed number increased
28. Flower and fruit abscission reduced	72. Seed quality altered
29. Fruit firmness increased	73. Seed set reduced
30. Fruit invertase level decreased	74. Seed weight increased
31. Fruit polygalacturonase level decreased	75. Senescence altered
32. Fruit ripening altered	76. Solids increased
33. Fruit ripening delayed	77. Starch level increased

34. Fruit solids increased	78. Starch metabolism altered
35. Fruit sugar profile altered	79. Starch reduced
36. Fruit sweetness increased	80. Steroidal glycoalkaloids reduced
37. Glyphosate tolerant	81. Sterols increased
38. Heat stable glucanase produced	82. Storage protein altered
39. Improved fruit quality	83. Sugar alcohol levels increased
40. Increased phosphorus	84. Thermostable protein produced
41. Increased protein levels	85. Tryptophan level increased
42. Lignin levels decreased	86. Tuber solids increased
43. Lysine level increased	87. Yield increased
44. Male sterile	

Table 2 – Part 6. Non-limiting examples of traits/phenotypes with herbicide tolerance properties

1. 2,4-D tolerant	11. Sulfonylurea tolerant
2. Chloroacetanilide tolerant	12. Northern corn leaf blight resistant
3. Fertility altered	13. Herbicide tolerant
4. Protein altered	14. Isoxazole tolerant
5. Lignin levels decreased	15. Chlorsulfuron tolerant
6. Methionine level increased	16. Glyphosate tolerant
7. Bromoxynil tolerant	17. Lepidopteran resistant
8. Metabolism altered	18. Phosphinothricin tolerant
9. Imidazole tolerant	19. Sulfonylurea tolerant
10. Imidazolinone tolerant	

Table 2 – Part 7. Non-limiting examples of traits/phenotypes with pest resistance properties

Legend

BR - Bacterial Resistant	NR - Nematode Resistant
FR - Fungal Resistant	VR - Viral Resistant
IR - Insect Resistant	

1. Agrobacterium resistant – BR	44. Ear mold resistant – FR
2. Alternaria resistant – FR	45. Erwinia carotovora resistant – BR
3. Alternaria daucii resistant – FR	46. European Corn Borer resistant – IR
4. Alternaria solani resistant – FR	47. Eyespot resistant – FR
5. AMV resistant – VR	48. Fall armyworm resistant – IR
6. Anthracnose resistant – FR	49. Fire blight resistant – BR
7. Aphid resistant – IR	50. Frogeye leaf spot resistant – FR
8. Apple scab resistant – FR	51. Fruit rot resistant – FR
9. Aspergillus resistant – FR	52. Fungal post-harvest resistant – FR
10. Bacterial leaf blight resistant – BR	53. Fungal resistant – FR
11. Bacterial resistant – BR	54. Fungal resistant general – FR
12. Bacterial soft rot resistant – BR	55. Fusarium dehliae resistant – FR
13. Bacterial soft rot resistant – VR	56. Fusarium resistant – FR
14. Bacterial speck resistant – BR	57. Geminivirus resistant – VR
15. BCTV resistant – VR	58. Gray lead spot resistant – FR
16. Black shank resistant – FR	59. Helminthosporium resistant – FR
17. BLRV resistant – VR	60. Hordothionin produced – BR
18. BNYYV resistant – VR	61. Insect predator resistant – IR
19. Botrytis cinerea resistant – FR	62. Insect resistant general – IR
20. Botrytis resistant – FR	63. Late blight resistant – FR
21. BPMV resistant – VR	64. Leaf blight resistant – FR
22. Brown spot resistant – FR	65. Leaf spot resistant – FR
23. BYDV resistant – VR	66. Lepidopteran resistant – IR
24. BYMV resistant – VR	67. Lesser cornstalk borer resistant – IR
25. CaMV resistant – VR	68. LMV resistant – VR
26. Cercospora resistant – FR	69. Loss of systemic resistance – VR
27. Clavibacter resistant – BR	70. Marssonina resistant – FR

28. Closterovirus resistant – BR	71. MCDV resistant – VR
29. CLRV resistant – VR	72. MCMV resistant – VR
30. CMV resistant – FR	73. MDMV resistant – VR
31. Coleopteran resistant – IR	74. MDMV-B resistant – VR
32. Colletotrichum resistant – FR	75. Mealybug wilt virus resistant – VR
33. Colorado potato beetle resistant – IR	76. Melampsora resistant – FR
34. Corn earworm resistant – IR	77. Melodgyne resistant – NR
35. Corynebacterium sepeidonicum resistant – BR	78. Meloidogyne resistant – NR
36. Cottonwood leaf beetle resistant – IR	79. Mexican Rice Borer resistant – IR
37. Cricconemella resistant – NR	80. Mycotoxin degradation – FR
38. Crown gall resistant – BR	81. Nepovirus resistant – VR
39. Cucumovirus resistant – VR	82. Northern corn leaf blight resistant – IR
40. Cylindrosporium resistant – FR	83. Nucleocapsid protein produced – VR
41. Disease resistant general – FR	84. Oblique banded leafhopper resistant – IR
42. Dollar spot resistant – FR	85. Oomycete resistant – FR
43. Downy mildew resistant – FR	86. Pathogenesis related proteins level increased – FR

Table 2 – Part 7. (continued) Non-limiting examples of traits/phenotypes with pest resistance properties

87. PEMV resistant – VR	116. SMV resistant – VR
88. PeSV Resistant – VR	117. Sod web worm resistant – IR
89. Phthora leaf beetle resistant – IR	118. Soft rot fungal resistant – FR
90. Phoma resistant – FR	119. Soft rot resistant – BR
91. Phytophthora resistant – FR	120. Southwestern corn borer resistant – IR
92. PLRV resistant – VR	121. SPFMV resistant – VR
93. Potyvirus resistant – VR	122. Sphaeropsis fruit rot resistant – FR
94. Powdery mildew resistant – FR	123. SqMV resistant – VR
95. PPV resistant – VR	124. SrMV resistant – VR
96. Pratylenchus vulnus resistant – NR	125. Streptomyces scabies resistant – BR
97. PRSV resistant – VR	126. Sugar cane borer resistant – IR
98. PRV resistant – VR	127. TEV resistant – VR
99. PSbMV resistant – VR	128. Thelaviopsis resistant – FR
100. Pseudomonas syringae resistant – BR	129. TMV resistant – FR
101. PSTV resistant – VR	130. Tobamovirus resistant – VR
102. PVX resistant – VR	131. ToMoV resistant – VR
103. PVY resistant – VR	132. ToMV resistant – VR
104. RBDV resistant – VR	133. TRV resistant – VR
105. Rhizoctonia resistant – FR	134. TSWV resistant – VR
106. Rhizoctonia solani resistant – FR	135. TVMV resistant – VR
107. Ring rot resistance – BR	136. TYLCV resistant – VR
108. Root-knot nematode resistant – NR	137. Venturia resistant – FR
109. Rust resistant – FR	138. Verticillium dahliae resistant – FR
110. SbMV resistant – VR	139. Verticillium resistant – FR
111. Sclerotinia resistant – FR	140. Western corn root worm resistant – IR
112. SCMV resistant – VR	141. WMV2 resistant – VR
113. SCYL V resistant – VR	142. WSMV resistant – VR
114. Septoria resistant – FR	143. ZYMV resistant – VR
115. Smut resistant – FR	

Table 2 – Part 8. Non-limiting examples of miscellaneous traits/phenotypes with properties

1. Antibiotic produced	31. Mycotoxin production inhibited
2. Antiprotease producing	32. Mycotoxin restored
3. Capable of growth on defined synthetic media	33. Non-lesion forming mutant
4. Carbohydrate metabolism altered	34. Novel protein produced
5. Cell wall altered	35. Oil quality altered
6. Cold tolerant	36. Peroxidase levels increased
7. Coleopteran resistant	37. Pharmaceutical proteins produced
8. Color altered	38. Phosphinothricin tolerant
9. Color sectors in seeds	39. Pigment metabolism altered
10. Colored sectors in leaves	40. Pollen visual marker

11. Constitutive expression of glutamine synthetase	41. Polyamine metablosim altered
12. Cre recombinase produced	42. Polymer produced
13. Dalapon tolerant	43. Recombinase produced
14. Development altered	44. Secondary metabolite increased
15. Disease resistant general	45. Seed color altered
16. Ethylene metabolism altered	46. Seed weight increased
17. Expression optimization	47. Selectable marker
18. Fenthion susceptible	48. Spectromycin resistant
19. Glucuronidase expressing	49. Sterile
20. Glyphosate tolerant	50. Sterols increased
21. Growth rate reduced	51. Sulfonylurea susceptible
22. Heavy metals sequestered	52. Syringomycin deficient
23. Hygromycin tolerant	53. Transposon activator
24. Inducible DNA modification	54. Transposon elements inserted
25. Industrial enzyme produced	55. Transposon inserted
26. Kanamycin resistant	56. Trifolitoxin producing
27. Lipase expressed in seeds	57. Trifolitoxin resistant
28. Methotrexate resistant	58. Virulence reduced
29. Modified growth characteristics	59. Visual marker
30. Mycotoxin deficient	60. Visual marker inactive

In a particular exemplification, “**producing an organism having a desirable trait**” includes an organism that is with respect to an organ or a part of an organ but not necessarily altered anywhere else.

By “**trait**” is meant any detectable parameter associated with an organism under a set of conditions. Examples of “detectable parameters” include the ability to produce a substance, the ability to not produce a substance, an altered pattern of (such as an increased or a decreased) ability to produce a substance, viability, non-viability, behaviour, growth rate, size, morphology or morphological characteristic,

In another embodiment, this invention is directed to a method of producing an organism having a desirable trait or a desirable improvement in a trait by: a) obtaining an initial population of organisms comprised of at least one starting organism, b) mutagenizing the population such that mutations occur throughout a substantial part of the genome of at least one initial organism, c) selecting at least one mutagenized organism having a desirable trait or a desirable improvement in a trait, and d) optionally repeating the method by subjecting one or more mutagenized organisms to a repetition of the method. A mutagenized organism having a desirable trait or a desirable improvement in a trait can be referred to as an “up-mutant”, and the associated mutation(s) contained in an up-mutant organism can be referred to as up-mutation(s).

[illegible]

Thus, in one embodiment, this invention is directed to a method of producing an organism having a desirable trait or a desirable improvement in a trait by: a) obtaining a starting population of organisms comprised of at least one starting organism, b) mutagenizing the population such that mutations occur throughout a substantial part of the genome of at least one starting organism, c) selecting at least one mutagenized organism having a desirable trait or a desirable improvement in a trait, and d) optionally repeating the method by subjecting one or more mutagenized organisms to a repetition of the method. A mutagenized organism having a desirable trait or a desirable improvement in a trait can be referred to as an "up-mutant", and the associated mutation(s) contained in an organism can be referred to as up-mutation(s).

substantial part of a starting population such that mutations occur throughout a least approximately some of at least one starting organism refers to mutagenizing at genes of a genome, all genes of a genome, or at least approximately 10% of the approximately 30% of approximately 20% of the genes of a genome, or at least genome, or at least approximately 40% of the genes

[illegible][illegible][illegible]

Figure 1 The effect of the number of nodes on the accuracy of the proposed method.

[illegible][illegible]

2) Introducing into the working cell or organism a plurality of traits (stacked traits), including selectively and differentially activatable traits. Serviceable traits for this purpose include traits conferred by genes and traits conferred by gene pathways.

3) Subjecting the working cell or organism to holistic monitoring.

4) Compiling the information obtained from steps 1) and 3), and processing &/or analyzing it to better understand the changes introduced into the working cell or organisms. Such data processing includes identifying correlations between and/or among the measured parameters.

5) Repeating any number or all of steps 2), 3), and 4).

This invention provides that molecules serviceable for introducing transgenic traits into a plant include all known genes and nucleic acids. By way of non-limiting exemplification, this invention specifically names any number &/or combination of genes listed herein or listed in any reference incorporated herein by reference . Furthermore, by way of non-limiting exemplification, this invention specifically names any number &/or combination of genes & gene pathways listed herein as well as in any reference incorporated by reference herein. This invention provides that molecules serviceable as detectable parameters include molecule, any enzyme, substrate thereof, product thereof, and any gene or gene pathway listed herein including in any figure or table herein as well as in any reference incorporated by reference herein.

This invention also relates generally to the field of nucleic acid engineering and correspondingly encoded recombinant protein engineering. More particularly, the invention relates to the directed evolution of nucleic acids and screening of clones containing the evolved nucleic acids for resultant activity(ies) of interest, such nucleic acid activity(ies) &/or specified protein, particularly enzyme, activity(ies) of interest.

Mutagenized molecules provided by this invention may have chimeric molecules and molecules with point mutations, including biological molecules that contain a carbohydrate, a

lipid, a nucleic acid, &/or a protein component, and specific but non-limiting examples of these include antibiotics, antibodies, enzymes, and steroidal and non-steroidal hormones.

This invention relates generally to a method of: 1) preparing a progeny generation of molecule(s) (including a molecule that is comprised of a polynucleotide sequence, a molecule that is comprised of a polypeptide sequence, and a molecules that is comprised in part of a polynucleotide sequence and in part of a polypeptide sequence), that is mutagenized to achieve at least one point mutation, addition, deletion, &/or chimerization, from one or more ancestral or parental generation template(s); 2) screening the progeny generation molecule(s) - preferably using a high throughput method - for at least one property of interest (such as an improvement in an enzyme activity or an increase in stability or a novel chemotherapeutic effect); 3) optionally obtaining &/or cataloguing structural &/or and functional information regarding the parental &/or progeny generation molecules; and 4) optionally repeating any of steps 1) to 3).

In a preferred embodiment, there is generated (e.g. from a parent polynucleotide template) - in what is termed "codon site-saturation mutagenesis" - a progeny generation of polynucleotides, each having at least one set of up to three contiguous point mutations (i.e. different bases comprising a new codon), such that every codon (or every family of degenerate codons encoding the same amino acid) is represented at each codon position. Corresponding to - and encoded by - this progeny generation of polynucleotides, there is also generated a set of progeny polypeptides, each having at least one single amino acid point mutation. In a preferred aspect, there is generated - in what is termed "amino acid site-saturation mutagenesis" - one such mutant polypeptide for each of the 19 naturally encoded polypeptide-forming alpha-amino acid substitutions at each and every amino acid position along the polypeptide. This yields - for each and every amino acid position along the parental polypeptide - a total of 20 distinct progeny polypeptides including the original amino acid, or potentially more than 21 distinct progeny polypeptides if additional amino acids are used either instead of or in addition to the 20 naturally encoded amino acids

Thus, in another aspect, this approach is also serviceable for generating mutants containing - in addition to &/or in combination with the 20 naturally encoded polypeptide-forming alpha-amino acids - other rare &/or not naturally-encoded amino acids and amino

acid derivatives. In yet another aspect, this approach is also serviceable for generating mutants by the use of - in addition to &/or in combination with natural or unaltered codon recognition systems of suitable hosts - altered, mutagenized, &/or designer codon recognition systems (such as in a host cell with one or more altered tRNA molecules).

In yet another aspect, this invention relates to recombination and more specifically to a method for preparing polynucleotides encoding a polypeptide by a method of *in vivo* re-assortment of polynucleotide sequences containing regions of partial homology, assembling the polynucleotides to form at least one polynucleotide and screening the polynucleotides for the production of polypeptide(s) having a useful property.

In yet another preferred embodiment, this invention is serviceable for analyzing and cataloguing - with respect to any molecular property (e.g. an enzymatic activity) or combination of properties allowed by current technology - the effects of any mutational change achieved (including particularly saturation mutagenesis). Thus, a comprehensive method is provided for determining the effect of changing each amino acid in a parental polypeptide into each of at least 19 possible substitutions. This allows each amino acid in a parental polypeptide to be characterized and catalogued according to its spectrum of potential effects on a measurable property of the polypeptide.

In another aspect, the method of the present invention utilizes the natural property of cells to recombine molecules and/or to mediate reductive processes that reduce the complexity of sequences and extent of repeated or consecutive sequences possessing regions of homology.

It is an object of the present invention to provide a method for generating hybrid polynucleotides encoding biologically active hybrid polypeptides with enhanced activities. In accomplishing these and other objects, there has been provided, in accordance with one aspect of the invention, a method for introducing polynucleotides into a suitable host cell and growing the host cell under conditions that produce a hybrid polynucleotide.

In another aspect of the invention, the invention provides a method for screening for biologically active hybrid polypeptides encoded by hybrid polynucleotides. The

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- c) detecting a mutagenized organism having an improved trait; and
- d) determining the nucleotide sequence of a gene that has been transferred into the detected organism.

D. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Exonuclease Activity. Figure 1 shows the activity of the enzyme exonuclease III. This is an exemplary enzyme that can be used to shuffle, assemble, reassemble, recombine, and/or concatenate polynucleotide building blocks. The asterisk indicates that the enzyme acts from the 3' direction towards the 5' direction of the polynucleotide substrate.

Figure 2. Generation of A Nucleic Acid Building Block by Polymerase-Based Amplification. Figure 2 illustrates a method of generating a double-stranded nucleic acid building block with two overhangs using a polymerase-based amplification reaction (e.g., PCR). As illustrated, a first polymerase-based amplification reaction using a first set of primers, F₂ and R₁, is used to generate a blunt-ended product (labeled Reaction 1, Product 1), which is essentially identical to Product A. A second polymerase-based amplification reaction using a second set of primers, F₁ and R₂, is used to generate a blunt-ended product (labeled Reaction 2, Product 2), which is essentially identical to Product B. These two products are then mixed and allowed to melt and anneal, generating a potentially useful double-stranded nucleic acid building block with two overhangs. In the example of Fig. 1, the product with the 3' overhangs (Product C) is selected for by nuclease-based degradation of the other 3 products using a 3' acting exonuclease, such as exonuclease III. Alternate primers are shown in parenthesis to illustrate serviceable primers may overlap, and additionally that serviceable primers may be of different lengths, as shown.

FIGURE 3. Unique Overhangs And Unique Couplings. Figure 3 illustrates the point that the number of unique overhangs of each size (e.g. the total number of unique overhangs composed of 1 or 2 or 3, etc. nucleotides) exceeds the number of unique couplings that can result from the use of all the unique overhangs of that size. For example, there are 4 unique 3' overhangs composed of a single nucleotide, and 4 unique 5' overhangs composed of a single nucleotide. Yet the total number of unique couplings that

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Figure 4 illustrates the fact that in order to assemble a total of “n” nucleic acid building blocks, “n-1” couplings are needed. Yet it is sometimes the case that the number of unique couplings available for use is fewer than the “n-1” value. Under these, and other, circumstances a stringent non-stochastic overall assembly order can still be achieved by performing the assembly process in sequential steps. In this example, 2 sequential steps are used to achieve a designed overall assembly order for five nucleic acid building blocks. In this illustration the designed overall assembly order for the five nucleic acid building blocks is: 5’-(#1-#2-#3-#4-#5)-3’, where #1 represents building block number 1, etc.

Figure 5 further illustrates the point that the number of unique overhangs of each size (here, e.g. the total number of unique overhangs composed of 2 nucleotides) exceeds the number of unique couplings that can result from the use of all the unique overhangs of that size. For example, there are 16 unique 3' overhangs composed of two nucleotides, and another 16 unique 5' overhangs composed of two nucleotides, for a total of 32 as shown. Yet the total number of couplings that are unique and not self-binding that can be made using all the 32 unique double-nucleotide 3' overhangs and double-nucleotide 5' overhangs is 12. Some apparently unique couplings have "identical twins" (marked in the same shading), which are visually obvious in this illustration. Still other overhangs contain nucleotide sequences that can self-bind in a palindromic fashion, as shown and labeled in this figure; thus they not contribute the high stringency to the overall assembly order.

Figure 6. Generation of an Exhaustive Set of Chimeric Combinations by Synthetic Ligation Reassembly. Figure 6 showcases the power of this invention in its ability to generate exhaustively and systematically all possible combinations of the nucleic acid building blocks designed in this example. Particularly large sets (or libraries) of progeny

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chimeric molecules can be generated. Because this method can be performed exhaustively and systematically, the method application can be repeated by choosing new demarcation points and with correspondingly newly designed nucleic acid building blocks, bypassing the burden of re-generating and re-screening previously examined and rejected molecular species. It is appreciated that, codon wobble can be used to advantage to increase the frequency of a demarcation point. In other words, a particular base can often be substituted into a nucleic acid building block without altering the amino acid encoded by progenitor codon (that is now altered codon) because of codon degeneracy. As illustrated, demarcation points are chosen upon alignment of 8 progenitor templates. Nucleic acid building blocks including their overhangs (which are serviceable for the formation of ordered couplings) are then designed and synthesized. In this instance, 18 nucleic acid building blocks are generated based on the sequence of each of the 8 progenitor templates, for a total of 144 nucleic acid building blocks (or double-stranded oligos). Performing the ligation synthesis procedure will then produce a library of progeny molecules comprised of yield of 8^{18} (or over 1.8×10^{16}) chimeras.

Figure 7. Synthetic genes from oligos:. According to one embodiment of this invention, double-stranded nucleic acid building blocks are designed by aligning a plurality of progenitor nucleic acid templates. Preferably these templates contain some homology and some heterology. The nucleic acids may encode related proteins, such as related enzymes, which relationship may be based on function or structure or both. Figure 7 shows the alignment of three polynucleotide progenitor templates and the selection of demarcation points (boxed) shared by all the progenitor molecules. In this particular example, the nucleic acid building blocks derived from each of the progenitor templates were chosen to be approximately 30 to 50 nucleotides in length.

Figure 8. Nucleic acid building blocks for synthetic ligation gene reassembly.

Figure 8 shows the nucleic acid building blocks from the example in Figure 7. The nucleic acid building blocks are shown here in generic cartoon form, with their compatible overhangs, including both 5' and 3' overhangs. There are 22 total nucleic acid building blocks derived from each of the 3 progenitor templates. Thus, the ligation synthesis procedure can produce a library of progeny molecules comprised of yield of 3^{22} (or over 3.1×10^{10}) chimeras.

Figure 9. Addition of Introns by Synthetic Ligation Reassembly. Figure 9 shows in generic cartoon form that an intron may be introduced into a chimeric progeny molecule by way of a nucleic acid building block. It is appreciated that introns often have consensus sequences at both termini in order to render them operational. It is also appreciated that, in addition to enabling gene splicing, introns may serve an additional purpose by providing sites of homology to other nucleic acids to enable homologous recombination. For this purpose, and potentially others, it may be sometimes desirable to generate a large nucleic acid building block for introducing an intron. If the size is overly large easily generating by direct chemical synthesis of two single stranded oligos, such a specialized nucleic acid building block may also be generated by direct chemical synthesis of more than two single stranded oligos or by using a polymerase-based amplification reaction as shown in Figure 2.

Figure 10. Ligation Reassembly Using Fewer Than All The Nucleotides Of An Overhang. Figure 10 shows that coupling can occur in a manner that does not make use of every nucleotide in a participating overhang. The coupling is particularly lively to survive (e.g. in a transformed host) if the coupling reinforced by treatment with a ligase enzyme to form what may be referred to as a "gap ligation" or a "gapped ligation". It is appreciated that, as shown, this type of coupling can contribute to generation of unwanted background product(s), but it can also be used advantageously increase the diversity of the progeny library generated by the designed ligation reassembly.

Figure 11. Avoidance of unwanted self-ligation in palindromic couplings. As mentioned before and shown in Figure 5, certain overhangs are able to undergo self-coupling to form a palindromic coupling. A coupling is strengthened substantially if it is reinforced by treatment with a ligase enzyme. Accordingly, it is appreciated that the lack of 5' phosphates on these overhangs, as shown, can be used advantageously to prevent this type of palindromic self-ligation. Accordingly, this invention provides that nucleic acid building blocks can be chemically made (or ordered) that lack a 5' phosphate group (or alternatively they can be remove – e.g. by treatment with a phosphatase enzyme such as a calf intestinal alkaline phosphatase (CIAP) – in order to prevent palindromic self-ligations in ligation reassembly processes.

Fig. 15. Holistic engineering of differentially activatable stacked traits in noveltransgenic plants using directed evolution and whole cell monitoring.

Fig. 16. Differential Activation of Selected Traits Can Be Achieved by Adjusting and Controlling the Environment of the Traits.

Fig. 17. Harvesting, Processing, Storage.

Fig. 18. Processing.

Fig. 19. Cellular Mutagenesis.

Figure 20. Differential Activation of Selected Precursor (Inactive) Gene Products.

Figure 21. Starting population comprised of an organism strain to be subjected to improvement or evolution in order to produce a resultant population comprised of an improved organism strain that has a desired trait.

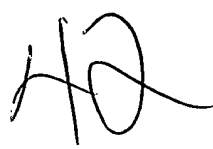
Figure 22. Starting population comprised of a genomic sequence to be subjected to improvement or evolution in order to produce a resultant population comprised of an improved genomic sequence that has a desired trait.

Fig. 23. Strain Improvement.

Fig. 24. Iterative Strain Improvement.

E. DEFINITIONS OF TERMS

In order to facilitate understanding of the examples provided herein, certain frequently occurring methods and/or terms will be described.



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The term "**antibody**", as used herein, refers to intact immunoglobulin molecules, as well as fragments of immunoglobulin molecules, such as Fab, Fab', (Fab')₂, Fv, and SCA fragments, that are capable of binding to an epitope of an antigen. These antibody fragments, which retain some ability to selectively bind to an antigen (*e.g.*, a polypeptide antigen) of the antibody from which they are derived, can be made using well known methods in the art (see, *e.g.*, **Harlow and Lane**, *supra*), and are described further, as follows.

- (1) An Fab fragment consists of a monovalent antigen-binding fragment of an antibody molecule, and can be produced by digestion of a whole antibody molecule with the enzyme papain, to yield a fragment consisting of an intact light chain and a portion of a heavy chain.
- (2) An Fab' fragment of an antibody molecule can be obtained by treating a whole antibody molecule with pepsin, followed by reduction, to yield a molecule consisting of an intact light chain and a portion of a heavy chain. Two Fab' fragments are obtained per antibody molecule treated in this manner.
- (3) An (Fab')₂ fragment of an antibody can be obtained by treating a whole antibody molecule with the enzyme pepsin, without subsequent reduction. A (Fab')₂ fragment is a dimer of two Fab' fragments, held together by two disulfide bonds.
- (4) An Fv fragment is defined as a genetically engineered fragment containing the variable region of a light chain and the variable region of a heavy chain expressed as two chains.
- (5) An single chain antibody ("SCA") is a genetically engineered single chain molecule containing the variable region of a light chain and the variable region of a heavy chain, linked by a suitable, flexible polypeptide linker.

The term "**Applied Molecular Evolution**" ("AME") means the application of an evolutionary design algorithm to a specific, useful goal. While many different library formats for AME have been reported for polynucleotides, peptides and proteins (phage, *lacI* and polysomes), none of these formats have provided for recombination by random cross-overs to deliberately create a combinatorial library.

A molecule that has a "**chimeric property**" is a molecule that is: 1) in part homologous and in part heterologous to a first reference molecule; while 2) at the same time being in part homologous and in part heterologous to a second reference molecule; without 3) precluding the possibility of being at the same time in part homologous and in part heterologous to still one or more additional reference molecules. In a non-limiting embodiment, a chimeric molecule may be prepared by assembling a reassortment of partial molecular sequences. In a non-limiting aspect, a chimeric polynucleotide molecule may be prepared by synthesizing the chimeric polynucleotide using plurality of molecular templates, such that the resultant chimeric polynucleotide has properties of a plurality of templates.

The term "**cognate**" as used herein refers to a gene sequence that is evolutionarily and functionally related between species. For example, but not limitation, in the human genome the human CD4 gene is the cognate gene to the mouse 3d4 gene, since the sequences and structures of these two genes indicate that they are highly homologous and both genes encode a protein which functions in signaling T cell activation through MHC class II-restricted antigen recognition.

A "**comparison window**," as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith (Smith and Waterman, *Adv Appl Math*, 1981; Smith and Waterman, *J Teor Biol*, 1981; Smith and Waterman, *J Mol Biol*, 1981;

Smith et al, *J Mol Evol*, 1981), by the homology alignment algorithm of Needleman (Needleman and Wuncsch, 1970), by the search of similarity method of Pearson (Pearson and Lipman, 1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected.

As used herein, the term "**complementarity-determining region**" and "**CDR**" refer to the art-recognized term as exemplified by the Kabat and Chothia CDR definitions also generally known as supervariable regions or hypervariable loops (Chothia and Lesk, 1987; Chothia et al, 1989; Kabat et al, 1987; and Tramontano et al, 1990). Variable region domains typically comprise the amino-terminal approximately 105-115 amino acids of a naturally-occurring immunoglobulin chain (e.g., amino acids 1-110), although variable domains somewhat shorter or longer are also suitable for forming single-chain antibodies.

"**Conservative amino acid substitutions**" refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are : valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

The term "**corresponds to**" is used herein to mean that a polynucleotide sequence is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence. In contradistinction, the term "complementary to" is used

purposes, typically 1 µg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 µl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 µg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a gel to isolate the desired fragment.

"Directional ligation" refers to a ligation in which a 5' end and a 3' end of a polynucleotide are different enough to specify a preferred ligation orientation. For example, an otherwise untreated and undigested PCR product that has two blunt ends will typically not have a preferred ligation orientation when ligated into a cloning vector digested to produce blunt ends in its multiple cloning site; thus, directional ligation will typically not be displayed under these circumstances. In contrast, directional ligation will typically displayed when a digested PCR product having a 5' *EcoR* I-treated end and a 3' *BamH* I-is ligated into a cloning vector that has a multiple cloning site digested with *EcoR* I and *BamH* I.

The term **"DNA shuffling"** is used herein to indicate recombination between substantially homologous but non-identical sequences, in some embodiments DNA shuffling may involve crossover via non-homologous recombination, such as via *cer/lox* and/or *flp/frt* systems and the like.

As used in this invention, the term **"epitope"** refers to an antigenic determinant on an antigen, such as a phytase polypeptide, to which the paratope of an antibody, such as an phytase-specific antibody, binds. Antigenic determinants usually consist of chemically active surface groupings of molecules, such as amino acids or sugar side chains, and can have specific three-dimensional structural characteristics, as well as specific charge characteristics. As used herein **"epitope"** refers to that portion of an antigen or other macromolecule capable of forming a binding interaction that interacts with the variable region binding body of an antibody. Typically, such binding interaction is manifested as an intermolecular contact with one or more amino acid residues of a CDR.

The terms "**fragment**", "**derivative**" and "**analog**" when referring to a reference polypeptide comprise a polypeptide which retains at least one biological function or activity that is at least essentially same as that of the reference polypeptide. Furthermore, the terms "fragment", "derivative" or "analog" are exemplified by a "pro-form" molecule, such as a low activity proprotein that can be modified by cleavage to produce a mature enzyme with significantly higher activity.

A method is provided herein for producing from a template polypeptide a set of progeny polypeptides in which a "**full range of single amino acid substitutions**" is represented at each amino acid position. As used herein, "**full range of single amino acid substitutions**" is in reference to the naturally encoded 20 naturally encoded polypeptide-forming alpha-amino acids, as described herein.

The term "**gene**" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

"**Genetic instability**", as used herein, refers to the natural tendency of highly repetitive sequences to be lost through a process of reductive events generally involving sequence simplification through the loss of repeated sequences. Deletions tend to involve the loss of one copy of a repeat and everything between the repeats.

The term "**heterologous**" means that one single-stranded nucleic acid sequence is unable to hybridize to another single-stranded nucleic acid sequence or its complement. Thus areas of heterology means that areas of polynucleotides or polynucleotides have areas or regions within their sequence which are unable to hybridize to another nucleic acid or polynucleotide. Such regions or areas are for example areas of mutations.

The term "**homologous**" or "**homeologous**" means that one single-stranded nucleic acid sequence may hybridize to a complementary single-stranded nucleic acid sequence. The degree of hybridization may depend on a number of factors including the amount of identity between the sequences and the hybridization conditions such as

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temperature and salt concentrations as discussed later. Preferably the region of identity is greater than about 5 bp, more preferably the region of identity is greater than 10 bp.

An immunoglobulin light or heavy chain variable region consists of a "framework" region interrupted by three hypervariable regions, also called CDR's. The extent of the framework region and CDR's have been precisely defined; see "Sequences of Proteins of Immunological Interest" (Kabat et al, 1987). The sequences of the framework regions of different light or heavy chains are relatively conserved within a specie. As used herein, a "**human framework region**" is a framework region that is substantially identical (about 85 or more, usually 90-95 or more) to the framework region of a naturally occurring human immunoglobulin. the framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDR's. The CDR's are primarily responsible for binding to an epitope of an antigen.

The benefits of this invention extend to "**commercial applications**" (or commercial processes), which term is used to include applications in commercial industry proper (or simply industry) as well as non-commercial commercial applications (e.g. biomedical research at a non-profit institution). Relevant applications include those in areas of diagnosis, medicine, agriculture, manufacturing, and academia.

The term "**identical**" or "**identity**" means that two nucleic acid sequences have the same sequence or a complementary sequence. Thus, "areas of identity" means that regions or areas of a polynucleotide or the overall polynucleotide are identical or complementary to areas of another polynucleotide or the polynucleotide.

The term "**isolated**" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or enzyme present in a living animal is not isolated, but the same polynucleotide or enzyme, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or enzymes could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

By "isolated nucleic acid" is meant a nucleic acid, e.g., a DNA or RNA molecule, that is not immediately contiguous with the 5' and 3' flanking sequences with which it normally is immediately contiguous when present in the naturally occurring genome of the organism from which it is derived. The term thus describes, for example, a nucleic acid that is incorporated into a vector, such as a plasmid or viral vector; a nucleic acid that is incorporated into the genome of a heterologous cell (or the genome of a homologous cell, but at a site different from that at which it naturally occurs); and a nucleic acid that exists as a separate molecule, e.g., a DNA fragment produced by PCR amplification or restriction enzyme digestion, or an RNA molecule produced by *in vitro* transcription. The term also describes a recombinant nucleic acid that forms part of a hybrid gene encoding additional polypeptide sequences that can be used, for example, in the production of a fusion protein.

As used herein "ligand" refers to a molecule, such as a random peptide or variable segment sequence, that is recognized by a particular receptor. As one of skill in the art will recognize, a molecule (or macromolecular complex) can be both a receptor and a ligand. In general, the binding partner having a smaller molecular weight is referred to as the ligand and the binding partner having a greater molecular weight is referred to as a receptor.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Sambrook et al, 1982, p. 146; Sambrook, 1989). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 μ g of approximately equimolar amounts of the DNA fragments to be ligated.

As used herein, "linker" or "spacer" refers to a molecule or group of molecules that connects two molecules, such as a DNA binding protein and a random peptide, and serves to place the two molecules in a preferred configuration, e.g., so that the random peptide can bind to a receptor with minimal steric hindrance from the DNA binding protein.

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As used herein, a **"nucleic acid molecule"** is comprised of at least one base or one base pair, depending on whether it is single-stranded or double-stranded, respectively. Furthermore, a nucleic acid molecule may belong exclusively or chimerically to any group of nucleotide-containing molecules, as exemplified by, but not limited to, the following groups of nucleic acid molecules: RNA, DNA, genomic nucleic acids, non-genomic nucleic acids, naturally occurring and not naturally occurring nucleic acids, and synthetic nucleic acids. This includes, by way of non-limiting example, nucleic acids associated with any organelle, such as the mitochondria, ribosomal RNA, and nucleic acid molecules comprised chimerically of one or more components that are not naturally occurring along with naturally occurring components.

Additionally, a **"nucleic acid molecule"** may contain in part one or more non-nucleotide-based components as exemplified by, but not limited to, amino acids and sugars. Thus, by way of example, but not limitation, a ribozyme that is in part nucleotide-based and in part protein-based is considered a **"nucleic acid molecule"**.

In addition, by way of example, but not limitation, a nucleic acid molecule that is labeled with a detectable moiety, such as a radioactive or alternatively a non-radioactive label, is likewise considered a **"nucleic acid molecule"**.

The terms **"nucleic acid sequence coding for"** or a **"DNA coding sequence of"** or a **"nucleotide sequence encoding"** a particular enzyme – as well as other synonymous terms – refer to a DNA sequence which is transcribed and translated into an enzyme when placed under the control of appropriate regulatory sequences. A **"promotor sequence"** is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The promoter is part of the DNA sequence. This sequence region has a start codon at its 3' terminus. The promoter sequence does include the minimum number of bases where elements necessary to initiate transcription at levels detectable above background. However, after the RNA polymerase binds the sequence and transcription is initiated at the start codon (3' terminus with a promoter), transcription proceeds downstream in the 3' direction. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping

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In one preferred embodiment, a **“specific nucleic acid molecule species”** is defined by its chemical structure, as exemplified by, but not limited to, its primary sequence. In another preferred embodiment, a specific **“nucleic acid molecule species”** is defined by a function of the nucleic acid species or by a function of a product derived from the nucleic acid species. Thus, by way of non-limiting example, a **“specific nucleic acid molecule species”** may be defined by one or more activities or properties attributable to it, including activities or properties attributable its expressed product.

The instant definition of “**assembling a working nucleic acid sample into a nucleic acid library**” includes the process of incorporating a nucleic acid sample into a vector-based collection, such as by ligation into a vector and transformation of a host. A description of relevant vectors, hosts, and other reagents as well as specific non-limiting examples thereof are provided hereinafter. The instant definition of “**assembling a working nucleic acid sample into a nucleic acid library**” also includes the process of incorporating a nucleic acid sample into a non-vector-based collection, such as by ligation to adaptors. Preferably the adaptors can anneal to PCR primers to facilitate amplification by PCR.

Accordingly, in a non-limiting embodiment, a **“nucleic acid library”** is comprised of a vector-based collection of one or more nucleic acid molecules. In another preferred embodiment a **“nucleic acid library”** is comprised of a non-vector-based collection of nucleic acid molecules. In yet another preferred embodiment a **“nucleic acid library”** is comprised of a combined collection of nucleic acid molecules that is in part vector-based

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An “**oligonucleotide**” (or synonymously an “**oligo**”) refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides may or may not have a 5' phosphate. Those that do not will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated. To achieve polymerase-based amplification (such as with PCR), a “**32-fold degenerate oligonucleotide that is comprised of, in series, at least a first homologous sequence, a degenerate N,N,G/T sequence, and a second homologous sequence**” is mentioned. As used in this context, “homologous” is in reference to homology between the oligo and the parental polynucleotide that is subjected to the polymerase-based amplification.

A coding sequence is "**operably linked to**" another coding sequence when RNA polymerase will transcribe the two coding sequences into a single mRNA, which is then

translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequences are ultimately processed to produce the desired protein.

As used herein the term "**parental polynucleotide set**" is a set comprised of one or more distinct polynucleotide species. Usually this term is used in reference to a progeny polynucleotide set which is preferably obtained by mutagenization of the parental set, in which case the terms "**parental**", "**starting**" and "**template**" are used interchangeably.

As used herein the term "**physiological conditions**" refers to temperature, pH, ionic strength, viscosity, and like biochemical parameters which are compatible with a viable organism, and/or which typically exist intracellularly in a viable cultured yeast cell or mammalian cell. For example, the intracellular conditions in a yeast cell grown under typical laboratory culture conditions are physiological conditions. Suitable *in vitro* reaction conditions for *in vitro* transcription cocktails are generally physiological conditions. In general, *in vitro* physiological conditions comprise 50-200 mM NaCl or KCl, pH 6.5-8.5, 20-45 °C and 0.001-10 mM divalent cation (e.g., Mg⁺⁺, Ca⁺⁺); preferably about 150 mM NaCl or KCl, pH 7.2-7.6, 5 mM divalent cation, and often include 0.01-1.0 percent nonspecific protein (e.g., BSA). A non-ionic detergent (Tween, NP-40, Triton X-100) can often be present, usually at about 0.001 to 2%, typically 0.05-0.2% (v/v). Particular aqueous conditions may be selected by the practitioner according to conventional methods. For general guidance, the following buffered aqueous conditions may be applicable: 10-250 mM NaCl, 5-50 mM Tris HCl, pH 5-8, with optional addition of divalent cation(s) and/or metal chelators and/or non-ionic detergents and/or membrane fractions and/or anti-foam agents and/or scintillants.

Standard convention (5' to 3') is used herein to describe the sequence of double stranded polynucleotides.

The term "**population**" as used herein means a collection of components such as polynucleotides, portions of polynucleotides or proteins. A "mixed population" means a collection of components which belong to the same family of nucleic acids or proteins

(i.e., are related) but which differ in their sequence (i.e., are not identical) and hence in their biological activity.

A molecule having a "**pro-form**" refers to a molecule that undergoes any combination of one or more covalent and noncovalent chemical modifications (e.g. glycosylation, proteolytic cleavage, dimerization or oligomerization, temperature-induced or pH-induced conformational change, association with a co-factor, etc.) en route to attain a more mature molecular form having a property difference (e.g. an increase in activity) in comparison with the reference pro-form molecule. When two or more chemical modification (e.g. two proteolytic cleavages, or a proteolytic cleavage and a deglycosylation) can be distinguished en route to the production of a mature molecule, the reference precursor molecule may be termed a "**pre-pro-form**" molecule.

As used herein, the term "**pseudorandom**" refers to a set of sequences that have limited variability, such that, for example, the degree of residue variability at another position, but any pseudorandom position is allowed some degree of residue variation, however circumscribed.

"**Quasi-repeated units**", as used herein, refers to the repeats to be re-assorted and are by definition not identical. Indeed the method is proposed not only for practically identical encoding units produced by mutagenesis of the identical starting sequence, but also the reassortment of similar or related sequences which may diverge significantly in some regions. Nevertheless, if the sequences contain sufficient homologies to be reassorted by this approach, they can be referred to as "quasi-repeated" units.

As used herein "**random peptide library**" refers to a set of polynucleotide sequences that encodes a set of random peptides, and to the set of random peptides encoded by those polynucleotide sequences, as well as the fusion proteins contain those random peptides.

As used herein, "**random peptide sequence**" refers to an amino acid sequence composed of two or more amino acid monomers and constructed by a stochastic or

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The term "**wild-type**" means that the polynucleotide does not comprise any mutations. A "wild type" protein means that the protein will be active at a level of activity found in nature and will comprise the amino acid sequence found in nature.

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In one aspect, this invention describes a new method to sequence DNA. The improvements over the existing DNA sequencing technologies are high speed, high throughput, no electrophoresis and gel reading artifacts due to the complete absence of an electrophoretic step, and no costly reagents involving various substitutions with stable isotopes. The invention utilizes the Sanger sequencing strategy and assembles the sequence information by analysis of the nested fragments obtained by basespecific chain termination via their different molecular masses using mass spectrometry, as for example, MALDI or ES mass spectrometry. A further increase in throughput can be obtained by introducing massmodifications in the oligonucleotide primer, chain-terminating nucleoside triphosphates and/or in the chainelongating nucleoside triphosphates, as well as using integrated tag sequences which allow multiplexing by hybridization of tag specific probes with mass differentiated molecular weights.

The present invention also pertains to a system for sequencing a genome. The system comprises a mechanism for obtaining nucleic acid material from a genome. The system also comprises a mechanism for constructing a clone library and one or more probe libraries. The constructing mechanism is in communication with the nucleic acid material from a genome. Additionally, the system comprises a mechanism for comparing said libraries to form comparisons. The comparing mechanism is in communication with the said libraries. The system also comprises a mechanism for combining the comparisons to construct a map of the clones relative to the genome. The said combining mechanism is in communication with the comparisons. Further, the system comprises a mechanism for determining the

sequence of the genome by means of said map. The said determining mechanism is in communication with said map. The present invention additionally pertains to a method for producing a gene of a genome.

An efficient method for sequencing large fragments of DNA is described. A subclone path through the fragment is first identified; the collection of subclones that define this path is then sequenced using transposon-mediated direct sequencing techniques to an extent sufficient to provide the complete sequence of the fragment.

Improved techniques are provided for DNA sequencing, and particularly for sequencing of the entire human genome. Different base-specific reactions are utilized to use different sets of DNA fragments from a piece of DNA of unknown sequence. Each of the different sets of DNA fragments has a common origin and terminates at a particular base along the unknown sequence. The molecular weight of the DNA fragments in each of the different sets is detected by a matrix assisted laser absorption mass spectrometer to determine the sequence of the different bases in the DNA. The methods and apparatus of the present invention provide a relatively simple and low cost technique which may be automated to sequence thousands of gene bases per hour, and eliminates the tedious and time consuming gel electrophoresis separation technique conventionally used to determine the masses of DNA fragments.

Processes and kits for simultaneously amplifying and sequencing nucleic acid molecules, and performing high throughput DNA sequencing are described.

A new contiguous genome sequencing method is described which allows the contiguous sequencing of a very long DNA without need to be subcloned. It uses the basic PCR technique but circumvents the usual need of this technique for the knowledge two primers for contiguous sequencing, enabling the knowledge of only one primer sufficient. The present invention makes it possible to PCR amplify a DNA adjacent to a known sequence with which one primer can be made without the knowledge of the second primer binding site present in the unknown sequence. The present invention could thus be used to contiguously sequence a very long DNA such as that contained in a YAC clone or a cosmid clone, without the need for subcloning smaller fragments, using the standard PCR technique. It can also be used to sequence a whole chromosome or genome without any need to subclone it.

Methods and means are provided for the massively parallel characterization of complex molecules and of molecular recognition phenomena with parallelism and

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The present invention pertains to a process for determining the DNA sequence of the genome of an organism. And more particularly, the invention relates to the sequencing of the entire human genome.

The invention relates to the massively parallel single molecule examination of associations or reactions between large numbers of first complex molecules, which may be diverse, and second single or plural probing molecules, which may or may not be diverse, with applications to biology, biotechnology, pharmacology, immunology, the novel field of cybernetic immunology, molecular evolution, cybernetic molecular evolution, genomics, comparative genomics, enzymology, clinical enzymology, pathology, medical research, and clinical medicine.

The present invention has applications in the area of polynucleotide sequence determination, including DNA sequencing.

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1. 2 SEQUENCING METHODS

1.2.1 Importance of DNA sequencing:

Current knowledge regarding gene structure, the control of gene activity and the function of cells on a molecular level all arose based on the determination of the base sequence of millions of DNA molecules. DNA sequencing is still critically important in research and for genetic therapies and diagnostics, (e.g., to verify recombinant clones and mutations).

DNA, a polymer of deoxyribonucleotides, is found in all living cells and some viruses. DNA is the carrier of genetic information, which is passed from one generation to the next by homologous replication of the DNA molecule. Information for the synthesis of all proteins is encoded in the sequence of bases in the DNA. DNA sequence information represents the information required for gene organization and regulation of most life forms. Accordingly, the development of reliable methodology for sequencing DNA has contributed significantly to an understanding of gene structure and function.

Since the genetic information is represented by the sequence of the four DNA building blocks deoxyadenosine- (dpA), deoxyguanosine- (dpG), deoxycytidine- (dpC) and deoxythyraine-5'-phosphate (dpT), DNA sequencing is one of the most fundamental technologies in molecular biology and the life sciences in general. The ease and the rate by which DNA sequences can be obtained greatly affects related technologies such as development and production of new therapeutic agents and new and useful varieties of plants and microorganisms via recombinant DNA technology. In particular, unraveling the DNA sequence helps in understanding human pathological conditions including genetic disorders, cancer and AIDS. In some cases, very subtle differences such as a one nucleotide deletion, addition or substitution can create serious, in some cases even fatal., consequences. Recently, DNA sequencing has become the core technology of the Human Genome Sequencing Project (e.g., J.E. Bishop and M. Waldholz, 1991, Genome: The Story of the Most Astonishing Scientific Adventure of Our Time - The Attempt to Map All the Genes in the Human Body, Simon & Schuster, New York). Knowledge of the complete human genome DNA sequence will certainly help to understand, to diagnose, to prevent and to treat human diseases. To be able to tackle successfully the determination of the approximately 3 billion base pairs of the human genome in a reasonable time frame

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In the chemical method, the DNA strand is isotropically labeled on one end, broken down into smaller fragments at sequence locations ending with a particular nucleotide (A, T, C, or G) by chemical means, and the fragments ordered based on this information. Base specific modifications result in a base specific cleavage of the radioactive or fluorescently labeled DNA fragment. After the DNA substrate is end labeled, it is subjected to chemical reactions designed to cleave the DNA at positions adjacent to a given base or bases. The labeled DNA fragments will, therefore, have a common labeled terminus while the unlabeled termini will be defined by the positions of chemical cleavage. This results in the generation of DNA fragments (four sets of nested fragments) which can be separated according to length by polyacrylamide gel electrophoresis (PAGE) and identified. Alternatively, unlabeled DNA fragments can be separated after complete restriction digestion and partial chemical cleavage of the DNA, and hybridized with probes homologous to a region near the region of the DNA to be sequenced. See, Church et al., Proc. Natl. Acad. Sci., 81:1991 (1984). After autoradiography, the sequence can be read directly since each band (fragment) in the gel originates from a base specific cleavage event. Thus, the fragment lengths in the four "ladders" directly translate into a specific position in the DNA sequence.

In the enzymatic method, the four base specific sets of DNA fragments are formed by starting with a primer/template system elongating the primer into the unknown DNA sequence area and thereby copying the template and synthesizing complementary strands using a DNA polymerase in the presence of chain-terminating reagents. The chain-terminating event is achieved by incorporating into the four separate reaction mixtures in addition to the four normal deoxynucleoside triphosphates, dATP, dGTP, dTTP and dCTP, only one of the chain-terminating dideoxynucleoside triphosphates, ddATP, ddGTP, ddTTP or ddCTP, respectively, in a

limiting small concentration. The incorporation of a ddNTP lacking the 3' hydroxyl function into the growing DNA strand by the enzyme DNA polymerase leads to chain termination through preventing the formation of a 3'-5'-phosphodiester bond by DNA polymerase. Due to the random incorporation of the ddNTPs, each reaction leads to a population of base specific terminated fragments of different lengths, which all together represent the sequenced DNA-molecule. The four sets of resulting fragments produce, after electrophoresis, four base specific ladders from which the DNA sequence can be determined.

In the enzymatic method, the following basic steps are involved:

(i) annealing an oligonucleotide primer to a suitable single or denatured double stranded DNA template; (ii) extending the primer with DNA polymerase in four separate reactions, each containing one α -labeled dNTP or ddNTP (alternatively a labeled primer can be used), a mixture of unlabeled dNTPs, and one chain-terminating dideoxynucleoside- 5'-triphosphate (ddNTP); (iii) resolving the four sets of reaction products, which include a distribution of DNA fragments having primer-defined 5' termini and differing dideoxynucleotides at the 3' termini, on a high resolution polyacrylamide-urea gel; and (iv) producing an auto radiographic image of the gel that can be examined to infer the DNA sequence. Alternatively, fluorescently labeled primers or nucleotides can be used to identify the reaction products. Known dideoxy sequencing methods utilize a DNA polymerase such as the Klenow fragment of *E. coli* DNA polymerase, a DNA polymerase from *Thermus aquaticus*, reverse transcriptase, a modified T7 DNA polymerase, or the Taq polymerase.

1. 2.2.3 Similarities, differences and other details of the two methods:

The two sequencing methods differ in the techniques employed to produce the DNA fragments, but are otherwise similar. In the Maxam-Gilbert method, four different base-specific reactions are performed on portions of the DNA molecules to be sequenced, to produce four sets of radiolabeled DNA fragments. These four fragment sets are each loaded in adjacent lanes of a polyacrylamide slab gel, and are separated by electrophoresis. Autoradiographic imaging of the pattern of the radiolabeled DNA bands in the gel reveals the relative size, corresponding to band mobilities, of the fragments in each lane, and the DNA sequence is deduced from this pattern.

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However, certain disadvantages inherent in the methodology of Guo and Wu, *supra*, limit its usefulness for the large scale sequencing of DNA. For example, this approach depends upon the selection of appropriate restriction enzymes which cleave at restriction sites in close proximity to particular *E. coli* exonuclease III endpoints, but not within the labeled DNA as this would result in two or more superimposed sequence ladders. The selection of appropriate restriction enzymes generally requires,

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1. 2.3 3'-hydroxy-protected and labeled nucleotides:

A modified nucleotide compound possessing two properties particularly useful for purposes of the present invention has been described by N. Williams and P.S. Coleman¹. This compound is 3'-O-(4-benzoyl)benzoyl adenosine 5'-triphosphate. This nucleotide bears a 3' protecting group linked via an ester function which should be susceptible to hydrolysis by appropriate chemical treatments. The protecting moiety is suitable for photoactivation, and this property was utilized by those investigators to probe the structure of mitochondrial F₁-ATPase, indicating that this analog will interact properly with at least some enzymes. Under appropriate circumstances, the protecting moiety may also serve as a label.

Very recently, B Canard and R.S. Sarfati have described similar nucleotides, here comprising all four nucleobases, with chemically removable 3'-hydroxyl protecting groups. Said protecting groups comprise various fluorescent dye moieties. These investigators have shown that these compounds may be added to appropriately primed polynucleotides by polymerases according to Watson-Crick base-pairing rules, and serve to terminate chain elongation in a manner which may be reversed by removal of said protecting groups by appropriate chemical treatments, admitting resumption of polymerization. These workers propose that such compounds may form the basis of a novel sequencing methodology availing stepping control by means of said removable protecting groups and detection of labels following their release from the nascent strand by appropriate chemical treatment. Such a method, while a potential advance over electrophoretic resolution methods, does not avail of great parallelism because only one molecule or an identical population of molecules may be sequenced at once (within a single vessel) by such a method, due to the release of the labeling moiety prior to detection, according to this proposed scheme. Further, this limitation requires that any attempt to avail of parallelism entail elaborate parallel fluidics. Low or no parallelism entails that stepping rate will be critical to the throughput attained with such a sequencing scheme. The results published by these authors suggests that the rate of chemical removal of 3'-hydroxy protecting groups (less than 90% removal after 10 minutes of treatment with 0.1M NaOH) will be unacceptably low for such an inherently serial sequencing scheme.

Additional references regarding such compounds and in most instances their properties as substrates for various enzymes including polymerases have been found

in the biological literature: Churchich, J.E.; 1995. Eur. J. Biochem., 231:736. Metzket, M.L.; Gibbs, R.A.; et al.; 1994. Nucleic Acids Research, 22:4259. Beabealashvilli, R.S.; Kukhanova, M.K.; et al.; 1986. Biochimica et Biophysica Acta, 868:136. Chidgeavadze, Z.G.; Kukhanova, M.K.; et al.; 1986. Biochimica et Biophysica Acta, 868:145. Hiratsuka, T; 1983. Biochimica et Biophysica Acta, 742:496. Jeng, S.J.; Guillory, R.J.; 1975. J. Supramolecular Structure, 3:448.

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1. 2.4 Related Base Addition Sequencing Schemes:

Various other investigators have also independently devised polynucleotide sequencing methodologies which depend on the addition of a polymerization terminating labeled nucleotide to a primed or elongated daughter strand on a polynucleotide sample with template dependent polynucleotide polymerases. Most, but not all, of these methods (referred to herein as previously disclosed base-addition sequencing schemes) avail nucleotide triphosphate monomers with some base-specific label which may be removed by some deprotection treatment. It must be emphasized that all of these other previously disclosed base-addition sequencing schemes examine not single molecules individually but rather large homogeneous populations of substantially identical molecules, wherein the observed signal used to identify label type originates from the totality of such a population of molecules rather than an individual molecule. It must be further emphasized that conventional usage does not generally reveal this distinction: phrases such a "a molecule" or "a sample molecule" refer not to an individual molecule considered separately or in isolation from other molecules including separately from other molecules of identical composition and structure, but to populations comprising millions or more molecules of identical structure. A careful reading of these prior disclosures reveals that these investigators are not working with samples consisting of single molecules but rather with samples comprising a plurality of identical molecules. In particular, even where these investigators do not (as is consistent with conventional usage) explicitly note this point, they take measures which would apply only to samples of pluralities of identical molecules, and do not take measures associated with working with single molecules.

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1.2.7 Electrophoresis

1. 2.7.1 Drawbacks and limitations of polyacrylamide gel electrophoresis (PAGE):

All methods discussed so far have one central step in common: polyacrylamide gel electrophoresis (PAGE). In many instances, this represents a major drawback and limitation for each of these methods. Preparing a homogeneous gel by polymerization, loading of the samples, the electrophoresis itself, detection of the sequence pattern (e.g., by autoradiography), removing the gel and cleaning the glass plates to prepare another gel are very laborious and time-consuming procedures.

Moreover, the whole process is error-prone, difficult to automate, and, in order to improve reproducibility and reliability, highly trained and skilled personnel are required.

In the case of radioactive labeling, autoradiography itself can consume from hours to days. In the case of fluorescent labeling, at least the detection of the sequencing bands is being performed automatically when using the laser-scanning devices integrated into commercial available DNA sequencers. One problem related to the fluorescent labeling is the influence of the four different base-specific fluorescent tags on the mobility of the fragments during electrophoresis and a possible overlap in the spectral bandwidth of the four specific dyes reducing the discriminating power between neighboring bands, hence, increasing the probability of sequence ambiguities. Artifacts are also produced by base-specific interactions with the polyacrylamide gel matrix (Frank and Köster, *Nucleic Acids Res.* -6, 2069 (1979)) and by the formation of secondary structures which result in "band compressions" and hence do not allow one to read the sequence. This problem has, in part, been overcome by using 7-deazadeoxyguanosine triphosphates (Barr et al., *Biotechniques* 4, 428 (1986)). However, the reasons for some artifacts and conspicuous bands are still under investigation and need further improvement of the gel electrophoretic procedure.

1. 2.7.2 Capillary zone electrophoresis (CZE):

A recent innovation in electrophoresis is capillary zone electrophoresis (CZE) (Jorgenson et al., J. Chromatography 352, 337 (1986); Gesteland et al., Nucleic Acids Res. 18, 1415-1419 (1990)) which, compared to slab gel electrophoresis (PAGE), significantly increases the resolution of the separation, reduces the time for an

electrophoretic run and allows the analysis of very small samples. Here, however, other problems arise due to the miniaturization of the whole system such as wall effects and the necessity of highly sensitive on-line detection methods. Compared to PAGE, another drawback is created by the fact that CZE is only a "one-lane" process, whereas in PAGE samples in multiple lanes can be electrophoresed simultaneously.

1. 2.7.3 DNA sequencing without the electrophoretic step:

Analysis methods have heretofore relied on electrophoretic separation and resolution of the products of Sanger or Maxam and Gilbert reactions according to the length of said products. Analysis thus suffers all of the limitations associated with electrophoresis including limited separation range (i.e. limited dynamic range, where separative resolution is related exponentially to fractional differences in molecular length), limitations on parallelism, time requirements, etc., despite much effort in improving these separative methodologies. With presently available equipment and trained personnel, sequencing the human genome would require about 100 years of total effort if no other sequencing projects were done. While very useful, the present sequencing methods are extremely tedious and expensive, yet require the services of highly skilled scientists. Moreover, these methods utilize hazardous chemicals and radioactive isotopes, which have inhibited their consideration and further development. Large scale sequencing projects, as that of the human genome, thus appear to be impractical using these well-established techniques.

In addition to being slow, the present DNA sequencing techniques involve a large number of cumbersome handling steps which are difficult to automate. Recent improvements include replacing the radioactive labels with fluorescent tags. These developments have improved the speed of the process and have removed some of the tedious manual steps, although present technology continues to employ the relatively slow gel electrophoresis technique for separating the DNA fragments.

Due to the severe limitations and problems related to having PAGE as an integral and central part in the standard DNA sequencing protocol, several methods have been proposed to do DNA sequencing without an electrophoretic step. One approach calls for hybridization or fragmentation sequencing (Bains, Biotechnology 10, 757-58 (1992) and Mirzabekov et al., FEBS Letters 256, 118-122 (1989)) utilizing the specific hybridization of known short oligonucleotides (e.g., octadeoxynucleotides which gives 65,536 different sequences) to a complementary DNA sequence. Positive

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1. 2.8 Mass Spectrometry

1. 2.8.1 Limitation in applying mass spectrometry due to the difficulty of volatilizing nucleic acids:

Therefore, "sequencing" has been limited to low molecular weight synthetic oligonucleotides by determining the mass of the parent molecular ion and through this, confirming the already known sequence, or alternatively, confirming the known sequence through the generation of secondary ions (fragment ions) via CID in an MS/MS configuration utilizing, in particular, for the ionization and volatilization, the method of fast atomic bombardment (FAB mass spectrometry) or plasma desorption (PD mass spectrometry). As an example, the application of FAB to the analysis of protected dimeric blocks for chemical synthesis of oligodeoxynucleotides has been described (Köster et al., Biochemical Environmental Mass Spectrometry 14, 111-116 (1987)).

1. 2.8.2 Two more ionization/desorption techniques (ES and MALDI):

Two more recent ionization/desorption techniques are electrospray/ion spray (ES) and matrix-assisted laser desorption/ionization (MALDI). ES mass spectrometry has been introduced by Fenn et al. J. Phys. Chem. 18, 4451-59 (1984); PCT Application No. WO 90/14148) and current applications are summarized in recent review articles (R.D. Smith et al., Anal. Chem. 62, 882-89 (1990) and B. Ardrey, Electrospray Mass Spectrometry, Spectroscopy Europe 4, 10-18 (1992)). The molecular weights of the tetradecanucleotide d(CATGCCATGGCATG) (Covey et al. "The Determination of Protein, Oligonucleotide and Peptide Molecular Weights by Ion spray Mass Spectrometry," Rapid Communications in Mass Spectrometry 3, 249- 256 (1988)), of the 21-mer d(AAATTGTGCACATCCTGCAGC) and without giving details of that of a tRNA with 76 nucleotides Methods in Enzymology 1. 23, "Mass Spectrometry" (McCloskey, editor), p. 425, 1990, Academic Press, New York) have been published. As a mass analyzer, a quadrupole is most frequently used. The determination of molecular weights in femtomole amounts of sample is very accurate due to the presence of multiple ion peaks which all could be used for the mass calculation.

MALDI mass spectrometry, in contrast, can be particularly attractive when a time-of-flight (TOF) configuration is used as a mass analyzer. The MALDI-TOF mass spectrometry has been introduced by Hillenkamp et al. ("Matrix Assisted UV-Laser

Desorption/Ionization: A New Approach to Mass Spectrometry of Large Biomolecules, Biological Mass Spectrometry (Burlingame and McCloskey, editors), Elsevier Science Publishers, Amsterdam, pp. 49-60, 1990.) Since, in most cases, no multiple molecular ion peaks are produced with this technique, the mass spectra, in principle, look simpler compared to ES mass spectrometry. Although DNA molecules up to a molecular weight of 410,000 daltons could be desorbed and volatilized (Williams et al., "Volatilization of High Molecular Weight DNA by Pulsed Laser Ablation of Frozen Aqueous Solutions," *Science*, 246, 1585-87 (1989)), this technique has so far only been used to determine the molecular weights of relatively small oligonucleotides of known sequence, e.g., oligothymidylic acids up to 18 nucleotides (Huth-Fehre et al., "Matrix- Assisted Laser Desorption Mass Spectrometry of Oligodeoxythymidylic Acids," *Rapid Communications in Mass Spectrometry*, 6, 209-13 (1992)) and a double-stranded DNA of 28 base pairs (Williams et al., "Time-of-Flight Mass Spectrometry of Nucleic Acids by Laser Ablation and Ionization from a Frozen Aqueous Matrix," *Rapid Communications in Mass Spectrometry*, 4, 348-351 (1990)). In one publication (Huth- Fehre et al., 1992 , supra), it was shown that a mixture of all the oligothymidylic acids from n=12 to n=18 nucleotides could be resolved.

1. 2.8.3 Producing fragments, separating by electrophoresis and using matrix method to sequence

In U.S. Patent No. 5,064,754, RNA transcripts extended by DNA both of which are complementary to the DNA to be sequenced are prepared by incorporating NTP's, dNTP's and, as terminating nucleotides, ddNTP's which are substituted at the 5'- position of the sugar moiety with one or a combination of the isotopes ^{12}C , ^{13}C , ^{14}C , ^1H , ^2H , ^3H , ^{16}O , ^{17}O and ^{18}O . The polynucleotides obtained are degraded to 3'- nucleotides, cleaved at the N-glycosidic linkage and the isotopically labeled 5'- functionality removed by periodate oxidation and the resulting formaldehyde species determined by mass spectrometry. A specific combination of isotopes serves to discriminate base-specifically between internal nucleotides originating from the incorporation of NTPs and dNTP's and terminal nucleotides caused by linking ddNTP's to the end of the polynucleotide chain. A series of RNA/DNA fragments is produced, and in one embodiment, separated by electrophoresis, and, with the aid of the so-called matrix method of analysis, the sequence is deduced.

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EPO Patent Applications No. 0360676 A1 and 0360677 A1 also describe Sanger sequencing using stable isotope substitutions in the ddNTP's such as D, ^{13}C , ^{15}N , ^{17}O , ^{18}O , ^{32}S , ^{33}S , ^{34}S , ^{36}S , ^{19}F , ^{35}Cl , ^{37}Cl , ^{79}Br , ^{81}Br and ^{127}I or function groups such as CF_3 or $\text{Si}(\text{CH}_3)_3$ at the base, the sugar or the alpha position of the triphosphate moiety according to chemical functionality. The Sanger sequencing reaction mixtures are separated by tube gel electrophoresis. The effluent is converted into an aerosol by the electrospray/thermospray nebulizer method and then atomized and ionize by a hot plasma (7000 to 8000°K) and analyzed by a simple mass analyzer. An instrument is proposed which enables one to automate the analysis of the Sanger sequencing reaction mixture consisting of tube electrophoresis, a nebulizer and a mass analyzer.

The application of mass spectrometry to perform DNA sequencing by the hybridization/fragment method (see above) has been recently suggested (Bains, "DNA Sequencing by Mass Spectrometry: Outline of a Potential Future Application, *Chimicaoggi* 2, 13-16 (1991)).

1. 2.9 Probes

1. 2.9.1 Using large arrays of nucleic acid probes on a substrate

Alternative techniques have been proposed for sequencing a nucleic acid. PCT patent Publication No. 92/10588, incorporated herein by reference for all purposes, describes one improved technique in which the sequence of a labeled, target nucleic acid is determined by hybridization to an array of nucleic acid probes on a substrate. Each probe is located at a positionally distinguishable location on the substrate. When the labeled target is exposed to the substrate, it binds at locations that contain complementary nucleotide sequences. Through knowledge of the sequence of the probes at the binding locations, one can determine the nucleotide sequence of the target nucleic acid. The technique is particularly efficient when very large arrays of nucleic acid probes are utilized.

Such arrays can be formed according to the techniques described in U.S. Patent No. 5,143,854 issued to Pirrung et al. See also U.S. application Serial No. 07/805,727, both incorporated herein by reference for all purposes.

1. 2.9.2 Employing sequencing by hybridization when the probes are shorter than the target

When the nucleic acid probes are of a length shorter than the target, one can employ a reconstruction technique to determine the sequence of the larger target based on affinity data from the shorter probes. See U.S. Patent No. 5,202,231 to Drmanac-et al., and PCT patent Publication No. 89/10977 to Southern. One technique for overcoming this difficulty has been termed sequencing by hybridization or SBH. For example, assume that a 12-mer target DNA 5'-AGCCTAGCTGAA is mixed with an array of all octanucleotide probes. If the target binds only to those probes having an exactly complementary nucleotide sequence, only five of the 65,536 octamer probes (3'-TCGGATCG, CGGATCGA, GGATCGAC, GATCGACT, and ATCGACTT) will hybridize to the target. Alignment of the overlapping sequences from the hybridizing probes reconstructs the complement of the original 12-mer target:

TCGGATCG

CGGATCGA

GGATCGAC

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The polymerase chain reaction (Mullis, K. et al., *Methods Enzymol.*, 155:335-350 1987) permits the selective in vitro amplification of a particular DNA region by mimicking the phenomena of in vivo DNA replication. Required reaction components are single stranded DNA, primers (oligonucleotide sequences complementary to the 5' and 3' ends of a defined sequence of the DNA template), deoxynucleotidetriphosphates and a DNA polymerase enzyme. Typically, the single stranded DNA is generated by heat denaturation of provided double strand DNA. The reaction buffers contain magnesium ions and co-solvents for optimum enzyme stability and activity.

The PCR amplification procedure has been used to sequence the DNA being amplified (e.g. "Introduction to the AmpliTaq Cycle Sequencing Kit Protocol", a booklet from Perkin Elmer Cetus Corporation). The DNA could be first amplified and then it could be sequenced using the two conventional DNA sequencing techniques. Modified methods for sequencing PCR-amplified DNA have also been developed (e.g. Bevan et al., "Sequencing of PCR-Amplified DNA" PCR Meth. App. 4:222 (1992)).

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[illegible]

However, amplification of the terminated fragments is linear in conventional cycle sequencing approaches. A recently developed method, called semi-exponential cycle sequencing shortens the time required and increases the extent of amplification obtained from conventional cycle sequencing by using a second reverse primer in the sequencing reaction. However, the reverse primer only generates additional template strands if it avoids being terminated prior to reaching the sequencing primer binding site. Needless to say, terminated fragments generated by the reverse primer can not serve as a sufficient template. Therefore, in practice, amplification by the semi-exponential approach is not entirely exponential. (Sarkat, G. and Bolander Mark E., Semi Exponential Cycle Sequencing Nucleic Acids Research, 1995, Vol. 23, No. 7, p. 1269-1270).

In addition to the foregoing limitations inherent in current sequencing techniques, the generation of DNA substrate molecules for each 300 to 500 nucleotides to be sequenced is presently required. Assuming no overlapping sequence between substrate molecules, the sequencing of both strands of an entire mammalian genome would, therefore, require the generation of at least 20 million DNA substrate molecules.

As pointed out above, current nucleic acid sequencing methods require relatively large amounts (typically about 1 g) of highly purified DNA template. Often, however, only a small amount of template DNA is available. Although amplifications may be performed, amplification procedures are typically time consuming, can be limited in the amount of amplified template produced and the amplified DNA must be purified prior to sequencing. A streamlined process for amplifying and sequencing DNA is needed, particularly to facilitate highthroughput nucleic acid sequencing.

[illegible][illegible][illegible][illegible]

Figure 10.10: The function $f(x) = \sin(x)$ and its derivative $f'(x) = \cos(x)$ on the interval $[0, 2\pi]$. The function $f(x)$ is shown in blue and $f'(x)$ is shown in red.

Figure 10.10: The function $f(x) = \sin(x)$ and its derivative $f'(x) = \cos(x)$ on the interval $[0, 2\pi]$. The function $f(x)$ is shown in blue and $f'(x)$ is shown in red.

Figure 10.10: The function $f(x) = \sin(x)$ and its derivative $f'(x) = \cos(x)$ on the interval $[0, 2\pi]$. The function $f(x)$ is shown in blue and $f'(x)$ is shown in red.

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[illegible]

The key requirement for this exponential increase of template DNA is the two oligo primers complementary to the ends of the sequence desired to be amplified, and oriented such that their 3' extension products proceed toward each other. If the sequence at both ends of the segment to be amplified is not known, complementary oligos cannot be made and standard PCR cannot be performed. The object of the present invention is to overcome the need for sequence information at both ends of the segment to be amplified, i.e. to provide a method which allows PCR to be performed when sequence is known for only a single region, and to provide a method for the

1. The first step is to identify the problem or question that needs to be addressed. This involves understanding the context and the specific requirements of the task.

1. 2.12.5 Large-scale sequencing through the generation of a subclone path

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chromosome 21 by radiation fusion hybrids," Mammalian Genome, vol. 5, no. 6, pp. 365-71, 1994), incorporated by reference.

1. 2.13.5 Inner product mapping

Inner product mapping (IPM) is a hybridization-based method for achieving high-throughput, high-resolution RH mapping of clones (M. W. Perlin and A. Chakravarti, "Efficient construction of high-resolution physical maps from yeast artificial chromosomes using radiation hybrids: inner product mapping," Genomics, vol. 18, pp. 283-289, 1993), incorporated by reference, that overcomes this barrier. Experimental data have established that IPM is a highly rapid, inexpensive, accurate, and precise large-scale long-range mapping method, particularly when preexisting RH maps are available, and that IPM can replace or complement more conventional short-range mapping methods.

1. 2.13.6 Obtaining improved mapping results

Improved mapping results can be obtained incrementally by gradually enlarging the data tables, a process which provides useful feedback to both experimentation and analysis. With additional RHs, the signal-to-noise characteristics of the clone profiles improve. This incremental process, and the relatively few RHs required for accurate mapping, follows the logarithmic number of the probes needed for IPM. For best mapping results, as many STS-typed RHs as feasible are used: with currently available high-throughput, robotically-assisted hybridization methods, the localization benefits of performing many filter hybridizations outweigh the relatively low experimentation costs. The incremental construction also highlights IPM's indirect inference of map location: STS-content mapping directly compares clones with STSs, and can not map small-insert clones against STSs which are insufficiently dense .

1. 2.13.7 Building accurate maps and partitioning data noise

IPM builds accurate maps from low-confidence data. IPM's partitioning of the experiments into two data tables of (A) clones vs. RHs and (B) RHs vs. STSs also partitions the data noise. Table B is formed from relatively noiseless PCR-based comparisons of STSs against RH DNA, and can thus accurately order and position the STS bins using combinatorial mapping procedures (M. Boehnke, "Radiation hybrid mapping by minimization of the number of obligate chromosome breaks," Genetic Analysis Workshop 7: Issues in Gene Mapping and the Detection of Major Genes.

of humans and other organisms, and making comparisons of genetic information between species. From these studies, diagnostic tests and pharmacological agents can be developed of great utility for preventing and treating human and other disease.

Disclosures of this type, yielded in a search, are:

Patent Number: Inventor Issued* US 5,302,509 Cheeseman, Peter C. 12 April 1994 WO 93/2134 Rosenthal., A; et al. 28 October 1993 DE 41 41 178 Al Ansorge, Wilhelm 16 June 1993 Wo 93/01583 Gibbs, Richard A.; et al. 18 March 1993 Wo 91/06678 Tsien, Roger Y.; et al. 16 May 1991 WO 90/13666 Garland, Peter B.; et al. 15 November 1990 Included in some of these above disclosures are descriptions of nucleotide triphosphates comprising removable fluorescent 3' protecting groups.

1.3 ALTERNATIVE SEQUENCING METHODS

The present invention provides an improved method of determining the nucleotide base sequence of DNA. In one embodiment, the method of the invention involves the preparation of a DNA substrate comprising a set of molecules, each having a template strand and a primer strand, wherein the 3' ends of the primer strands of the molecules terminate at about the same nucleotide position on the template strands of the molecules within each set. Preferably, the template and primer strands of the molecules are of unequal lengths wherein the 3' ends of the primer strands of the molecules terminate at about the same nucleotide position on the template strands of the molecules within each set. DNA synthesis is induced to obtain labeled reaction products comprising newly synthesized DNA complementary to the template strands using the 3' ends of the primer strands to prime DNA synthesis, labeled nucleoside triphosphates, at least one modified nucleoside triphosphate, and preferably, a suitable chain terminator, wherein the modified nucleoside triphosphate is selected to substantially protect newly synthesized DNA from cleavage. Thereafter, the labeled reaction products are cleaved at one or more selected sites to obtain labeled DNA fragments wherein newly synthesized DNA is substantially protected from cleavage by the incorporation of the modified nucleotide. The labeled DNA fragments obtained in the preceding step are separated and their nucleotide base sequence is identified by suitable means. The advantages of the present invention over prior art methods will become apparent after consideration of the accompanying drawings and the following detailed description of the invention.

1. The first step is to identify the problem or goal. This involves understanding the current situation, identifying the problem, and setting a clear goal.

When combined with a detection means, the process can be used to detect and/or quantitate a particular nucleic acid sequence where only small amounts of template are available and fast and accurate sequence data acquisition is desirable. For example, when combined with a detection means, the process is useful for sequencing unknown genes or other nucleic acid sequences and for diagnosing or monitoring certain diseases or conditions, such as genetic diseases, chromosomal abnormalities, genetic predispositions to certain diseases (e.g. cancer, obesity, atherosclerosis) and pathogenic (e.g. bacterial., viral., fungal., protistal) infections. Further, when double stranded DNA molecules are used as the starting material., the instant process

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1.3.2 Base-specific Reactions Used on DNA fragments from a piece of an unknown sequence

In accordance with the present invention, there is also provided a method and apparatus for determining the sequence of the bases in DNA by measuring the molecular mass of each of the DNA fragments in mixtures prepared by either the Maxam-Gilbert or Sanger-Coulson techniques. The fragments are preferably prepared as in these standard techniques, although the fragments need not be tagged with radioactive tracers. These standard procedures produce from each section of DNA to be sequenced four separate collections of DNA fragments, each set containing fragments terminating at only one or two of the four bases. In the Maxam-Gilbert method, the four separated collections contain fragments terminating at G, both G and A, both C and T, or C positions, respectively. Each of these collections is sequentially loaded into an ultraviolet laser desorption mass spectrometer, and the mass spectrum of each collection is recorded and stored in the memory of a computer. These spectra are recorded under conditions such that essentially no fragmentation occurs in the mass spectrometer, so that the mass of each ion measured corresponds to the molecular weight of one of the DNA fragments in the collection, plus a proton in the positive ion spectrum, and minus a proton in the negative ion spectrum. Spectra obtained from the four spectra are compared using a computer algorithm, and the location of each of the four bases in the sequence is unambiguously determined.

It is also possible, in principle, to obtain the DNA sequence from a single mass spectrum obtained from a more complex single mixture containing all possible fragments, but both the resolution and mass accuracy required are much higher than in the preferred method described above. As a result the accuracy of the DNA sequence obtained from the single spectrum method will generally be inferior, and the gain in raw sequence speed will be counterbalanced by the need for more repetitions to assure accuracy of the sequence.

The DNA fragments to be analyzed are dissolved in a liquid solvent containing a matrix material. Each sample is radiated with a UV laser beam at a wavelength of between 260 nm to 560 nm, and pulses of from 1 to 20 ns pulsewidth.

It is an objective of the present invention to provide a method and apparatus for the rapid and accurate sequencing of human genome and other DNA material.

It is a further objective of the present invention to provide an instrument and method which are relatively simple to operate, relatively low in cost, and which may be automated to sequence thousands of gene bases per hour.

It is a further objective of the present invention to obtain much faster and more accurate DNA sequence data by eliminating the gel electrophoresis separation technique used in conventional DNA sequencing methods to determine the masses of the DNA fragments in a mixture.

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1.3.3 Sequencing Through Exposure To Immobilized Probes Of Shorter Length

According to one embodiment of the invention, a target oligonucleotide is exposed to a large number of immobilized probes of shorter length. The probes are collectively referred to as an "array." In the method, one identifies whether a target nucleic acid is complementary to a probe in the array by identifying first a core probe having high affinity to the target, and then evaluating the binding characteristics of all probes with a single base mismatch as compared to the core probe. If the single base mismatch probes exhibit a characteristic binding or affinity pattern, then the core probe is exactly complementary to at least a portion of the target nucleic acid.

The method can be extended to sequence a target nucleic acid larger than any probe in the array by evaluating the binding affinity of probes that can be termed "left" and "right" extensions of the core probe. The correct left and right extensions of the core are those that exhibit the strongest binding affinity and/or a specific hybridization pattern of single base mismatch probes.

The binding affinity characteristics of single base mismatch probes follow a characteristic pattern in which probe/target complexes with mismatches on the 3' or 5' termini are more stable than probe/target complexes with internal mismatches. The process is then repeated to determine additional left and right extensions of the core probe to provide the sequence of a nucleic acid target.

In some embodiments, such as in diagnostics, a target is expected to have a particular sequence. To determine if the target has the expected sequence, an array of probes is synthesized that includes a complementary probe and all or some subset of all single base mismatch probes. Through analysis of the hybridization pattern of the target to such probes, it can be determined if the target has the expected sequence and, if not, the sequence of the target may optionally be determined.

Kits for analysis of nucleic acid targets are also provided by virtue of the present invention. According to one embodiment, a kit includes an array of nucleic acid probes. The probes may include a perfect complement to a target nucleic acid.

The probes also include probes that are single base substitutions of the perfect complement probe. The kit may include one or more of the A, C, T, G, and/or U substitutions of the perfect complement. Such kits will have a variety of uses,

1.3.4 Sequencing Contiguously, Without The Need For Fragmenting And Sub-Cloning The DNA

The present invention also enables the amplification of a DNA adjacent to a known sequence using the PCR, without the knowledge of the sequence for a second primer.

The present primary invention also provides a new method for sequencing a contiguously very long DNA sequence using the PCR technique, thereby enabling contiguous genomic sequencing. It will avoid the need for mapping or sub-cloning of shorter DNA fragments from haploid genomes such as the bacterial genomes. This method can be used on very large DNA inserts into vectors such as the YAC. Thus, diploid genomes can be sequenced without any further need to sub-clone from the YAC clones. The cloned inserts can be of any length, of several million nucleotides. Alternatively, wherever purified chromosomes are available, this method can be directly applied to sequence the whole chromosome without any need to fragment the chromosome or obtain YAC clones from the chromosome. This method can also be used on whole unpurified genomes with appropriate modifications to account for the allelic variations of the two alleles present on the two chromosomes. In essence, using the method of the present invention, one can generate contiguous genomic sequence information in a manner not possible with any other known protocol using PCR.

The extended invention that enables the sequencing of an unknown region of very long DNA (e.g. genomic DNA) of totally unknown sequence would also find many applications in biology and medicine. For instance, it can be used to physically "map" a chromosome or genome. It would, for example, enable the production of an inventory of many about 500 nucleotide long sequences and the exact primer associated with each of them. This method would also enable the cloning of the amplified DNA sequences from arbitrary regions from a genomic DNA without the need for breaking down the DNA. Using appropriately longer partly fixed primers (as the second primers), very long DNA pieces (several kilobases long) could be amplified and cloned by using this method.

1.3.4.1 PCR Technique with 1 Primer

In one embodiment, the present invention enables the amplification of a DNA stretch using the PCR procedure with the knowledge of only one primer. Using this basic method, the present invention describes a procedure by which a very long DNA

of the order of millions of nucleotides can be sequenced contiguously, without the need for fragmenting and sub-cloning the DNA. In this method, the general PCR technique is used, but the knowledge of only one primer is sufficient, and the knowledge of the other primer is derived from the statistics of the distributions of oligonucleotide sequences of specified lengths.

Present DNA sequencing methods using the separation of DNA fragments on a gel has a limitation of resolving the products of length up to about 1000 nucleotides. Thus, in a single step, the sequence of a DNA fragment up to a length of only about 1000 nucleotides can be obtained by the two conventional DNA sequencing methods. A DNA sequence of a few nucleotides up to many thousand nucleotides can be amplified by the PCR procedure. Thus the PCR procedure can be combined with the DNA sequencing procedure successfully.

A primer is usually of length twelve nucleotides and longer. Let the sequence of one primer is known in a long DNA sequence from which the DNA sequence is to be worked out. From this primer sequence, a specific sequence of four nucleotides occurs statistically at an average distance of 256 nucleotides. It has been worked out by Senapathy that a particular sequence of four characters would occur anywhere from zero distance up to about 1500 characters with a 99.9% probability (P. Senapathy, "Distribution and repetition of sequence elements in eukaryotic DNA: New insights by computer aided statistical analysis," Molecular Genetics (Life Sciences Advances), 7:53-65 (1988)). The mean distance for such an occurrence is 256 characters and the median is 180 characters. Similarly, a 5 nucleotide long specific sequence will occur at a mean distance of 1024 characters, with 99.99% of them occurring within 6000 characters from the first primer. The median distance for the occurrence of a 5-nucleotide specific sequence is ~730 nucleotides. Similarly, a particular 6 nucleotide long sequence will occur at a mean distance of 4096 nucleotides and a median distance of ~2800 nucleotides. A primer of known length, say length 14 can be prepared with a known sequence of 6 characters and the rest of the sequence being random in sequence. It means that any of the four nucleotides can occur at the "random" sequence locations. With a fixed 5, 6 or 7 nucleotide sequence within the second primer, a primer of length 12-18 can be prepared with high specificity of binding.

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Thus, in one aspect, the invention is directed to a method to sequence a fragment of DNA, said fragment typically having a length of more than about 30 kb. The method comprises the following steps.

A composition comprising said vector containing the inserted fragment is then randomly sheared, such as by sonication, to obtain subfragments of approximately 3 kb. The length of the subfragments is appropriate to the transposon-mediated directed sequencing method that will ultimately be applied. The 3 kb length is an approximation; it is intended only as an order of magnitude. Generally speaking, subfragments of 2-5 kb are susceptible to this approach.

The subfragments are then inserted into host cloning vectors to obtain a library of subclones. These host cloning vectors are ideally of minimal size, containing only a selectable marker, an origin of replication, and appropriate insertion sites for the subfragments. The desirability of minimizing the available plasmid DNA in the performance of transposon-mediated sequencing is described by Strathmann, et al. (supra).

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sequences adjacent the inserts to proceed into the insert to approximately this distance. For a 1 x coverage of the original fragment, the number of subclones required can be calculated by dividing the length of the original fragment by the intended sequencing distance--i.e., by approximately 400- 450.

There should also be sufficient subclones in the library so that when the complete sequence of each is determined, the coverage of the original fragment will be about 7-8 x. This provides, as described below, a high probability that every nucleotide present in the fragment will be present in the library. This number can, of course, be determined by multiplying the length of the fragment by 7 or 8 and dividing by the length of the subfragments generated.

It is preferable to assure that all of the subclones in the library contain pieces of the original fragment. This can be done by recovering only those subclones that hybridize to the fragments.

A sufficient portion of one of the ends of each recovered subclone containing fragment-derived DNA is then sequenced and this sequence information is placed into a searchable database. The database is searched for subclones that contain subfragments with nucleotide sequences matching those that characterize the host vector that accommodated the original fragment. To the extent that these subfragments also contain sequence from the original fragment, that sequence must be at one or the other end of the original fragment. This illustrates why the efficiency of the method is improved by introducing a prescreening step which eliminates any subclones which do not contain portions of the original fragment. If the prescreening has been done, these subclones contain oligonucleotide sequence from either end of the original fragment. The identified subclones are recovered.

1.3.5.1 "Second End" Sequence

A partial sequence of each of the identified subclones is determined from the opposite end of the subfragment insert from that originally placed in the database. This provides "second end" sequence information concerning sequence further removed from the end of the original fragment. This information is then used to search the database in order to identify subclones containing nucleotide sequence that matches this second end sequence. Such subclones are likely to represent regions of the original fragment that are farther removed from the ends and provide further progress in constructing a path across the fragment. These subclones are recovered as

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1.3.6 Improvements in high speed, high throughput, no required electrophoresis (and, thus, no gel reading artifacts due to the complete absence of an electrophoretic step)

The invention also describes a new method to sequence DNA. The improvements over the existing DNA sequencing technologies include high speed, high throughput, no required electrophoresis (and, thus, no gel reading artifacts due to the complete absence of an electrophoretic, step), and no costly reagents involving various substitutions with stable isotopes. The invention utilizes the Sanger sequencing strategy and assembles the sequence information by analysis of the nested fragments obtained by base-specific chain termination via their different molecular masses using mass spectrometry, for example, MALDI or ES mass spectrometry. A further increase in throughput can be obtained by introducing mass modifications in the oligonucleotide primer, the chain-terminating nucleoside triphosphates and/or the chain- elongating nucleoside triphosphates, as well as using integrated tag sequences which allow multiplexing by hybridization of tag specific probes with mass differentiated molecular weights.

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Used within appropriate procedures, single molecule examination methods can enable molecular parallelism.

Molecular parallelism may be applied to the examination of the composition of complex molecules (including co-polymers of natural or of synthetic origin) or to determinations of interactions between large numbers of molecules. The former case may be applied to genome-scale sequencing methods. The latter case may be applied to rapid determination of molecular complementarity, with applications in (biological or non-biological) affinity characterization, immunological study, clinical pathology, molecular evolution (e.g. in vitro evolution), and the construction of a cybernetic immune system as well as prostheses based thereupon. In both cases, molecular recognition phenomena are observed with molecular parallelism.

Note that within said affinity characterization applications, both kinetics of both binding association and dissociation, and binding equilibria, may be examined. Kinetics may be examined by observing the rates of occupation of appropriate sites or diverse populations thereof by some homogenous or heterogeneous sample, and the rates of vacancy formation from occupied sites. Equilibria constants may be determined by observing the proportion (number of occupied sites divided by number of total sites) of sites occupied under equilibrium conditions, with greater quantitative confidence yielded by, for example, examining more binding sites.

Sequencing of polynucleotide molecules may be effected by the (preferably end-wise) immobilization of a library of such molecules to a surface at a density convenient for detection, which will vary according to the detection methodology availed. Several methods capable of effecting such immobilization will be obvious to those skilled in the arts of recombinant DNA technology and molecular biology,

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1.4 EXEMPLARY GENOMIC CHARACTERIZATION METHODS

1.4.1 Employing Mass Spectrometry To Analyze The Sanger Sequencing Reaction Mixtures

In one embodiment, this invention describes an improved method of sequencing DNA. In particular, this invention employs mass spectrometry to analyze the Sanger sequencing reaction mixtures.

In Sanger sequencing, four families of chain-terminated fragments are obtained. The mass difference per nucleotide addition is 289.19 for dpC, 313.21 for dpA, 329.21 for dpG and 304.2 for dpT, respectively.

1.4.1.1 Mass Modified

In one embodiment, through the separate determination of the molecular weights of the four base-specifically terminated fragment families, the DNA sequence can be assigned via superposition (e.g., interpolation) of the molecular weight peaks of the four individual experiments. In another embodiment, the molecular weights of the four specifically terminated fragment families can be determined simultaneously by MS, either by mixing the products of all four reactions run in at least two separate reaction vessels (i.e., all run separately, or two together, or three together) or by running one reaction having all four chain-terminating nucleotides (e.g., a reaction mixture comprising dTTP, ddTTP, dATP, ddATP, dCTP, ddCTP, dGTP, ddGTP) in one reaction vessel. By simultaneously analyzing all four base-specifically terminated reaction products, the molecular weight values have been, in effect, interpolated. Comparison of the mass difference measured between fragments with the known masses of each chain-terminating nucleotide allows the assignment of sequence to be carried out. In some instances, it may be desirable to mass modify, as discussed below, the chain-terminating nucleotides so as to expand the difference in molecular weight between each nucleotide. It will be apparent to those skilled in the art when mass-modification of the chain-terminating nucleotides is desirable and can depend, for instance, on the resolving ability of the particular spectrometer employed. By way of example, it may be desirable to produce four chain-terminating nucleotides, ddTTP, ddCTP, ddATP and ddGTP where ddCTP, ddATP and ddGTP have each been mass-modified so as to have molecular weights resolvable from one another by the particular spectrometer being used.

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The terms chain-elongating nucleotides and chain-terminating nucleotides are well known in the art. For DNA, chain-elongating nucleotides include 2'-deoxyribonucleotides and chain-terminating nucleotides include 2', 3'-dideoxyribonucleotides. For RNA, chain-elongating nucleotides include ribonucleotides and chain-terminating nucleotides include 3'-deoxyribonucleotides. The term nucleotide is also well known in the art. For the purposes of this invention, nucleotides include nucleoside mono-, di-, and triphosphates. Nucleotides also include modified nucleotides such as phosphorothioate nucleotides.

Since mass spectrometry is a serial method, in contrast to currently used slab gel electrophoresis which allows several samples to be processed in parallel, in another embodiment of this invention, a further improvement can be achieved by multiplex mass spectrometric DNA sequencing to allow simultaneous sequencing of more than one DNA or RNA fragment. As described in more detail below, the range of about 300 mass units between one nucleotide addition can be utilized by employing either mass modified nucleic acid sequencing primers or chain-elongating and/or terminating nucleoside triphosphates so as to shift the molecular weight of the base-specifically terminated fragments of a particular DNA or RNA species being sequenced in a predetermined manner. For the first time, several sequencing reactions can be mass spectrometrically analyzed in parallel. In yet another embodiment of this invention, multiplex mass spectrometric DNA sequencing can be performed by mass modifying the fragment families through specific oligonucleotides (tag probes) which hybridize to specific tag sequences within each of the fragment families. In another embodiment, the tag probe can be covalently attached to the individual and specific tag sequence prior to mass spectrometry.

1.4.1.2 Mass Spectrometer Formats Used (MALDI, ES, ICR, Fourier Transform)

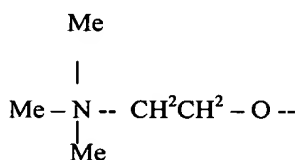
Preferred mass spectrometer formats for use in the invention are matrix assisted laser desorption ionization (MALDI), electrospray (ES), ion cyclotron resonance (ICR) and Fourier Transform. For ES, the samples, dissolved in water or in a volatile buffer, are injected either continuously or discontinuously into an atmospheric pressure ionization interface (API) and then mass analyzed by a quadrupole. The generation of multiple ion peaks which can be obtained using ES mass spectrometry can increase the accuracy of the mass determination. Even more

the laser energy can be tuned to the corresponding energy of the charge-transfer wavelength and, thus, a specific desorption off the solid support can be initiated. Those skilled in the art will recognize that several combinations can serve this purpose and that the donor functionality can be either on the solid support or coupled to the nested Sanger DNA/RNA fragments or vice versa.

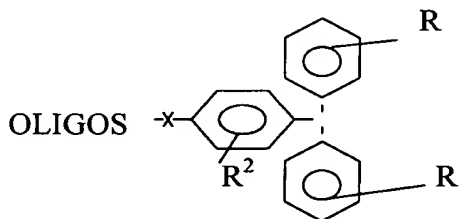
In yet another approach, the temporary linkage L-L' can be generated by homolytically forming relatively stable radicals. As described herein, a combination of the approaches using charge-transfer complexes and stable organic radicals is shown. Here, the nested Sanger DNA/RNA fragments are captured via the formation of a charge transfer complex. Under the influence of the laser pulse, desorption (as discussed above) as well as ionization will take place at the radical position. In other examples described herein, under the influence of the laser pulse, the L-L' linkage will be cleaved and the nested Sanger DNA/RNA fragments desorbed and subsequently ionized at the radical position formed. Those skilled in the art will recognize that other organic radicals can be selected and that, in relation to the dissociation energies needed to homolytically cleave the bond between them, a corresponding laser wavelength can be selected (see e.g. *Reactive Molecules* by C. Wenstrup, John Wiley & Sons, 1984). In yet another approach, the nested Sanger DNA/RNA fragments are captured via Watson-Crick base pairing to a solid support-bound oligonucleotide complementary to either the sequence of the nucleic acid primer or the tag oligonucleotide sequence. The duplex formed will be cleaved under the influence of the laser pulse and desorption can be initiated. The solid support-bound base sequence can be presented through natural oligoribo- or oligodeoxyribonucleotide as well as analogs (e.g. thio-modified phosphodiester or phosphotriester backbone) or employing oligonucleotide mimetics such as PNA analogs (see e.g. Nielsen et al., *Science*, 254, 1497 (1991)) which render the base sequence less susceptible to enzymatic degradation and hence increases overall stability of the solid support-bound capture base sequence. With appropriate bonds, L-L', a cleavage can be obtained directly with a laser tuned to the energy necessary for bond cleavage. Thus, the immobilized nested Sanger fragments can be directly ablated during mass spectrometric analysis.

1.4.1.3.1 Conditioning

Prior to mass spectrometric analysis, it may be useful to "condition" nucleic acid molecules, for example to decrease the laser energy required for volatilization, to minimize fragmentation or to otherwise increase the sensitivity of mass spectrometric detection. For example, nucleic acids can be "conditioned" by adding positive or negative charges, i.e. charge tags (CTs). CTs increase the mass spectrometer detection sensitivity by increasing the degree of ionization during the mass spectrometric (e.g. MALDI) process. A CT can be linked either to the external 3' or 5' position or internally e.g. at the 2' position or at the base, e.g. at C-5 in uracil, C-5 methyl group of thymine, C-5 at cytosine, at C⁷ or C⁸ guanine, adenine and hypoxanthine or at the phosphate ester moiety. Charge tags, CTs, can function molecules with permanent (i.e. pH-independent) ionization, such as:



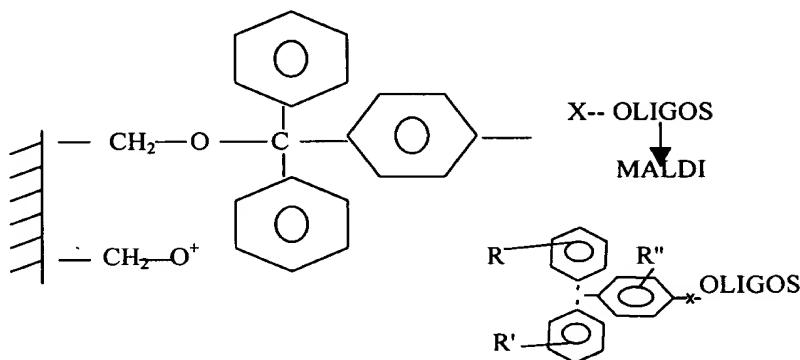
or molecules which generate a positive charge upon MALDI and which are stabilized by delocalization of the positive charge by mesomeric effects in unsaturated and/or aromatic systems such as:



wherein, R, R', R' = H, or Al (wherein Al = e.g. lower alkyl, methyl, ethyl, propyl), NO₂, CN, CO₂H, CO₂ active ester, or halogen; and X = -O-, -NH-, -S-, C=O, OCO either in the para or meta position.

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In an alternative scheme, the trityl group is used to anchor the oligonucleotide to a solid support via the tertiary carbon and this bond is cleaved during mass spectrometry (e.g. MALDI), leaving a positive charge on the desorbing and high vacuum flying oligonucleotide.



One of skill in the art can readily appreciate several variations to the schemes described above. In addition to employing the charge tag array alone, one of skill in the art can employ a charge tag array in conjunction with another conditioning means. Particularly preferred means to be used in conjunction with the CT include treating the phosphodiester bond with trialkylsilyl halides or the phosphomonothiodiester bond with alkyl iodides to render the polyanionic backbone neutral.

1.4.1.3.1.1 Modification of phosphodiester Backbone of Nucleic Acid Molecule

Another example of conditioning is modification of the phosphodiester backbone of the nucleic acid molecule (e.g. cation exchange), which can be useful for eliminating peak broadening due to a heterogeneity in the cations bound per nucleotide unit. In addition, a nucleic acid molecule can be contacted with an alkylating agent such as alkyl iodide, iodoacetamide, β -iodoethanol, or 2,3-epoxy-1-propanol, the monothio phosphodiester bonds of a nucleic acid molecule can be transformed into a phosphotriester bond. Likewise, phosphodiester bonds may be transformed to uncharged derivatives employing trialkylsilyl chlorides. Further conditioning involves incorporating nucleotides which reduce sensitivity for depurination (fragmentation during MS) such as N7- or N9-deazapurine nucleotides, or RNA building blocks or using oligonucleotide triesters or incorporating phosphorothioate functions which are alkylated or employing oligonucleotide mimetics such as PNA.

Modification of the phosphodiester backbone can be accomplished by, for example, using alpha-thio modified nucleotides for chain elongation and termination. With alkylating agents such as alkyl iodides, iodoacetamide, β -iodoethanol, 2,3-epoxy-1-propanol, the monothio phosphodiester bonds of the nested Sanger fragments are transformed into phosphotriester bonds. Multiplexing by mass modification in this case is obtained by mass-modifying the nucleic acid primer (UP) or the nucleoside triphosphates at the sugar or the base moiety. To those skilled in the art, other modifications of the nested Sanger fragments can be envisioned. In one embodiment of the invention, the linking chemistry allows one to cleave off the so-purified nested DNA enzymatically, chemically or physically. By way of example, the L-L' chemistry can be of a type of disulfide bond (chemically cleavable, for example, by mercaptoethanol or dithioerythrol), a biotin/streptavidin system, a heterobifunctional derivative of a trityl ether group (Koster et al., "A Versatile Acid-Labile Linker for Modification of Synthetic Biomolecules," Tetrahedron Letters 31, 7095 (1990)) which can be cleaved under mildly acidic conditions, a levulinyl group cleavable under almost neutral conditions with a hydrazinium/acetate buffer, an arginine-arginine or lysine-lysine bond cleavable by an endopeptidase enzyme like trypsin or a pyrophosphate bond cleavable by a pyrophosphatase, a photocleavable bond which can be, for example, physically cleaved and the like. Optionally, another cation exchange can be performed prior to mass spectrometric analysis. In the instance that an enzyme-cleavable bond is utilized to immobilize the nested fragments, the enzyme used to cleave the bond can serve as an internal mass standard during MS analysis.

1.4.1.3.2 Purification Process

The purification process and/or ion exchange process can be carried out by a number of other methods instead of, or in conjunction with, immobilization on a solid support. For example, the base-specifically terminated products can be separated from the reactants by dialysis, filtration (including ultrafiltration), and chromatography.

Likewise, these techniques can be used to exchange the cation of the phosphate backbone with a counter-ion which reduces peak broadening.

The base-specifically terminated fragment families can be generated by standard Sanger sequencing using the Large Klenow fragment of E. coli DNA polymerase I, by Sequenase, Taq DNA polymerase and other DNA polymerases

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1.4.1.4 Data Analysis (ES, MALDI)

The highly purified, four base-specifically terminated DNA or RNA fragment families are then analyzed with regard to their fragment lengths via determination of their respective molecular weights by MALDI or ES mass spectrometry.

For ES, the samples, dissolved in water or in a volatile buffer, are injected either continuously or discontinuously into an atmospheric pressure ionization interface (API) and then mass analyzed by a quadrupole. With the aid of a computer program, the molecular weight peaks are searched for the known molecular weight of the nucleic acid primer (UP) and determined which of the four chain terminating nucleotides has been added to the UP. This represents the first nucleotide of the unknown sequence. Then, the second, the third, the n^{th} extension product can be identified in a similar manner and, by this, the nucleotide sequence is assigned. The generation of multiple ion peaks which can be obtained using ES mass spectrometry can increase the accuracy of the mass determination.

1.4.1.5 Process for Multiplex Mass Spectrometric DNA Sequencing Employing Mass Modified Reagents

As illustrative embodiments of this invention, three different basic processes for multiplex mass spectrometric DNA sequencing employing the described mass-modified reagents are described below:

A) Multiplexing by the use of mass-modified nucleic acid primers (UP) for Sanger DNA or RNA sequencing,

B) Multiplexing by the use of mass-modified nucleoside triphosphates as chain elongators and/or chain terminators for Sanger DNA or RNA sequencing, and

C) Multiplexing by the use of tag probes which specifically hybridize to tag sequences which are integrated into part of the four Sanger DNA/RNA base-specifically terminated fragment families. Mass modification here can be achieved as described hereing, or alternately, by designing different oligonucleotide sequences having the same or different length with unmodified nucleotides which, in a predetermined way, generate appropriately differentiated molecular weights.

The process of multiplexing by mass-modified nucleic acid primers (UP) is illustrated by way of example herein for mass analyzing four different DNA clones simultaneously. The first reaction mixture is obtained by standard Sanger DNA

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sequencing having unknown DNA fragment 1 (clone 1) integrated in an appropriate vector (e.g., M13mpl8), employing an unmodified nucleic acid primer UP^0 , and a standard mixture of the four unmodified deoxynucleoside triphosphates, $dNTP^0$ and with 1/10th of one of the four dideoxynucleoside triphosphates, $ddNTP$. A second reaction mixture for DNA fragment 2 (clone 2) is obtained by employing a mass-modified nucleic acid primer UP^1 and, as before, the four unmodified nucleoside triphosphates, 0 $dNTP$, containing in each separate Sanger reaction 1/10th of the chain-terminating unmodified dideoxynucleoside triphosphates $ddNTP$. In the other two experiments, the four Sanger reactions have the following compositions: DNA fragment 3 (clone 3), UP^2 , $dNTP^0$, $ddNTP^0$ and DNA fragment 4 (clone 4), UP^3 , $dNTP^0$, $ddNTP^0$. For mass spectrometric DNA sequencing, all base-specifically terminated reactions of the four clones are pooled and mass analyzed. The various mass peaks belonging to the four dideoxy-terminated (e.g., ddT-terminated) fragment families are assigned to specifically elongated and ddT-terminated fragments by searching (such as by a computer program) for the known molecular ion peaks of UP^0 , UP^1 , UP^2 and UP^3 extended by either one of the four dideoxynucleoside triphosphates, $UP^0 ddN^0$, $UP^1 ddN^0$, $UP^2 ddN^0$ and $UP^3 - ddN^0$. In this way, the first nucleotides of the four unknown DNA sequences of clone 1 to 4 are determined. The process is repeated, having memorized the molecular masses of the four specific first extension products, until the four sequences are assigned. Unambiguous mass/sequence assignments are possible even in the worst case scenario in which the four mass-modified nucleic acid primers are extended by the same dideoxynucleoside triphosphate, the extension products then being, for example, $UP^0 ddT$, $UP^1 -ddT$, $UP^2 -ddT$ and $UP^3 -ddT$, which differ by the known mass increment differentiating the four nucleic acid primers. In another embodiment of this invention, an analogous technique is employed using different vectors containing, for example, the SP6 and/or T7 promoter sequences, and performing transcription with the nucleic acid primers UP^0 , UP^1 , UP^2 and UP^3 and either an RNA polymerase (e.g., SP6 or T7 RNA polymerase) with chain-elongating and terminating unmodified nucleoside triphosphates NTP^0 and 3'- $dNTP^0$. Here, the DNA sequence is being determined by Sanger RNA sequencing.

Illustrated herein is the process of multiplexing by mass-modified chain-elongating or/and terminating nucleoside triphosphates in which three different DNA

Nature 344, 358 (1990) and Thuong et al. "Oligonucleotides Attached to Intercalators, Photoreactive and Cleavage Agents" in F. Eckstein, Oligonucleotides and Analogues A Practical Approach, IRL Press, Oxford 1991, 283-306).

The DNA sequence is unraveled again by searching for the lowest molecular weight molecular ion peak corresponding to the known UP⁰-tag sequence/tag probe molecular weight plus the first extension product, e.g., ddT⁰, then the second, the third, etc.

In a combination of the latter approach with the previously described multiplexing processes, a further increase in multiplexing can be achieved by using, in addition to the tag probe/tag sequence interaction, mass-modified nucleic acid primers and/or mass-modified deoxynucleoside, dNTP⁰⁻ⁱ, and/or dideoxynucleoside triphosphates, ddNTP⁰⁻ⁱ. Those skilled in the art will realize that the tag sequence/tag probe multiplexing approach is not limited to Sanger DNA sequencing generating nested DNA fragments with DNA polymerases. The DNA sequence can also be determined by transcribing the unknown DNA sequence from appropriate promoter-containing vectors (see above) with various RNA polymerases and mixtures of NTP⁰⁻¹ 3' dNTP⁰⁻¹, thus generating nested RNA fragments.

In yet another embodiment of this invention, the mass-modifying functionality can be introduced by a two or multiple step process. In this case, the nucleic acid primer, the chain-elongating or terminating nucleoside triphosphates and/or the tag probes are, in a first step, modified by a precursor functionality such as azido, -N₃, or modified with a functional group in which the R in XR is H thus providing temporary functions, e.g., but not limited to -OH, -NH₂, -NHR, -SH, -NCS, -OCO(CH₂)_rCOOH (r = 1-20), -NHCO(CH₂)_rCOOH (r = 1-20), -OSO₂OH, -OCO(CH₂)_r' (r = 1-20), -OP(O-Alkyl)N(Alkyl)₂. These less bulky functionalities result in better substrate properties for the enzymatic DNA or RNA synthesis reactions of the DNA sequencing process. The appropriate mass-modifying functionality is then introduced after the generation of the nested base-specifically terminated DNA or RNA fragments prior to mass spectrometry. Several examples of compounds which can serve as mass-modifying functionalities are depicted herein without limiting the scope of this invention.

1.4.1.6 Kits for Sequencing Nucleic Acid by Mass Spectrometry

Another aspect of this invention concerns kits for sequencing nucleic acids by mass spectrometry which include combinations of the above-described sequencing

terminating nucleotide; (iv) a second DNA polymerase, which has a relatively high affinity towards the chain terminating nucleotide; and (v) an RE that nicks the primer recognition/cleavage site.

Steps 1) - 3) can be sequentially performed for an appropriate number of times (cycles) to obtain the desired amount of amplified sequencing ladders. As with the PCR based process, the quantity of the base specifically terminated fragment desired dictates how many cycles are performed. Preferably, less than 50 cycles, more preferably less than about 40 cycles and most preferably about 20 to 30 cycles are performed.

The amplified sequencing ladders obtained as described above, can be separated and detected and/or quantitated using well established methods, such as polyacrylamide gel electrophoresis (PAGE), or capillary zone electrophoresis (CZE) (Jorgenson et al., J. Chromatography 352, 337 (1986); Gesteland et al., Nucleic Acids Res. L8, 1415-1419 (1990)); or direct blotting electrophoresis (DBE) (Beck and Pohl, EMBO J, vol. 3: Pp. 2905-2909 (1984)) in conjunction with, for example, colorimetry, fluorimetry, chemiluminescence and radioactivity.

Dye-terminator chemistry can be employed in the combined amplification and sequencing reaction to enable the simultaneous generation of forward and reverse sequence ladders, which can be separated based on the streptavidin-biotin system when one biotinylated primer is provided.

Depicted herein is a scheme for the combined amplification and sequencing using two polymerases and dye-labeled chain terminating nucleotide (ddNTP) for detection and two reverse oriented primers. A means of separation for the simultaneously generated forward and reverse sequence ladders is shown. Step A represents the exponential amplification of a target sequence by the polymerase with a low affinity for ddNTPs. One of the sequence specific oligonucleotide primers is biotinylated. Step B represents the generation of a sequence ladder either from the original template or the simultaneously generated amplification product carried out by the polymerase with a high affinity for ddNTPs. After completion of the reaction, the products are incubated with a streptavidin coated solid support (Step C). Biotinylated forward sequencing products and reverse products hybridized to the forward template are immobilized. In order to obtain readable sequence information, the forward and reverse sequence ladders are separated in Step D. The immobilized strands are

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disruption (e.g. sonication or nebulization)); and e) RNA or RNA transcripts like mRNAs.

The nucleic acid to be amplified and sequenced can be obtained from virtually any biological sample. As used herein, the term "biological sample" refers to any material obtained from any living source (e.g. human, animal, plant, bacteria, fungi, protist, virus). Examples of appropriate biological samples for use in the instant invention include: solid materials (e.g. tissue, cell pellets, biopsies) and biological fluids (e.g. urine, blood, saliva, amniotic fluid, mouth wash, spinal fluid). The nucleic acid to be amplified and sequenced can be provided by unpurified whole cells, bacteria or virus.

Alternatively, the nucleic acid can first be purified from a sample using standard techniques, such as: a) cesium chloride gradient centrifugation; b) alkaline lysis with or without RNase treatment; c) ion exchange chromatography; d) phenol/chloroform extraction; e) isolation by hybridization to bound oligonucleotides; f) gel electrophoresis and elution; alcohol precipitation and h) combinations of the above.

As used herein, the phrases "chain-elongating nucleotides" and "chain-terminating nucleotides" are used in accordance with their art recognized meaning. For example, for DNA, chain-elongating nucleotides include 2'- deoxyribonucleotides (e.g. dATP, dCTP, dGTP and dTTP) and chain-terminating nucleotides include 2', 3'-dideoxyribonucleotides, (e.g. ddATP, ddCTP, ddGTP, ddTTP). For RNA, chain-elongating nucleotides include ribonucleotides (e.g., ATP, CTP, GTP and UTP) and chain-terminating nucleotides include 3'-deoxyribonucleotides (e.g. 3'dA, 3'dC, 3'dG and 3'dU). A complete set of chain elongating nucleotides refers to dATP, dCTP, dGTP and dTTP. The term "nucleotide" is also well known in the art. For the purposes of this invention, nucleotides include nucleoside mono-, di-, and triphosphates. Nucleotides also include modified nucleotides, such as phosphorothioate nucleotides and deazapurine nucleotides. A complete set of chain-elongating nucleotides refers to four different nucleotides that can hybridize to each of the four different bases comprising the DNA template.

If the amplified sequencing ladders are to be detected by mass spectrometric analysis, it may be useful to "condition" nucleic acid molecules, for example to decrease the laser energy required for volatilization and/or to minimize fragmentation.

Conditioning is preferably performed while the sequencing ladders are immobilized. An example of conditioning is modification of the phosphodiester backbone of the nucleic acid molecule (e.g. cation "change"), which can be useful for eliminating peak broadening due to a heterogeneity in the cations bound per nucleotide unit.

Contacting a nucleic acid molecule, which contains an -thio-nucleoside-triphosphate during polymerization with an alkylating agent such as alkyl iodide, iodoacetamide, - iodoethanol, or 2,3-epoxy-1-propanol, the monothio phosphodiester bonds of a nucleic acid molecule can be transformed into a phosphotriester bond. Further conditioning involves incorporating nucleotides which reduce sensitivity for depurination (fragmentation during MS), e.g. a purine analog such as N7- or N9-deazapurine nucleotides, and partial RNA containing oligodeoxynucleotide to be able to remove the unmodified primer from the amplified and modified sequencing ladders by RNase or alkaline treatment. In DNA sequencing using fluorescent detection and gel electrophoretic separation, the N7 deazapurine nucleotides reduce the formation of secondary structure resulting in band compression from which no sequencing information can be generated.

1.4.2.2 The Use of Two Polymerase Enzymes Each Having Different Affinities for the Chain Terminating Nucleotides

Critical to the novel process of the invention is the use of appropriate amounts of two different polymerase enzymes, each having a different affinity for the particular chain terminating nucleotide, so that polymerization by the enzyme with relatively low affinity for the chain terminating nucleotide leads to amplification whereas the enzyme with relatively high affinity for the chain terminating nucleotide terminates the polymerization and yields sequencing products. Preferably about 0.5 to about 3 units of polymerase is used in the combined amplification and chain termination reaction. Most preferably about 1 to 2 units is used. Particularly preferred polymerases for use in conjunction with PCR or other thermal amplification process are thermostable polymerases, such as Taq DNA polymerase (Boehringer Mannheim), AmpliTaq FS DNA polymerase (Perkin-Elmer), Deep Vent (exo-), Vent, Vent (exo-) and Deep Vent DNA polymerases (New England Biolabs), Thermo Sequenase (Amersham) or exo(-) Pseudococcusfuriosus (Pfu) DNA polymerase (Stratagene, Heidelberg Germany). AmpliTaq, Ultman, 9 degree Nm, Tth, Hot Tub,

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Oligonucleotide primers, for use in the invention, can be designed based on knowledge of the 5' and/or 3' regions of the nucleotide sequence to be amplified and sequenced, e.g., insert flanking regions of cloning and sequencing vectors (such as M13, pUC, phagemid, costaid). Optionally, at least one primer used in the chain extension and termination reaction can be linked to a solid support to facilitate purification of amplified product from primers and other reactants, thereby increasing yield or to separate the Sanger ladders from the sense and antisense template strand where simultaneous amplification-sequencing of both a sense and antisense strand of the template DNA has been performed.

Examples of appropriate solid supports include beads (silica gel, controlled pore glass, magnetic beads, Sephadex/Sepharose beads, cellulose beads, etc.), capillaries, flat supports such as glass fiber filters, glass surfaces, metal surfaces (steel, gold, silver, aluminum, and copper), plastic materials or membranes (polyethylene, polypropylene, polyamide, polyvinylidenedifluoride) or beads in pits of flat surfaces such as wafers (e.g. silicon wafers), with or without filter plates.

Immobilization can be accomplished, for example, based on hybridization between a capture nucleic acid sequence, which has already been immobilized to the support and a complementary nucleic acid sequence, which is also contained within the nucleic acid molecule containing the nucleic acid sequence to be detected. So that hybridization between the complementary nucleic acid molecules is not hindered by

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the support, the capture nucleic acid can include a spacer region of at least about five nucleotides in length between the solid support and the capture nucleic acid sequence. The duplex formed will be cleaved under the influence of the laser pulse and desorption can be initiated. The solid support-bound base sequence can be presented through natural oligoribo- or oligodeoxyribo- nucleotide as well as analogs (e.g. thio-modified phosphodiester or phosphotriester backbone) or employing oligonucleotide mimetics such as PNA analogs (see e.g. Nielsen et al., Science, 254, 1497 (1991)) which render the base sequence less susceptible to enzymatic degradation and hence increases overall stability of the solid support-bound capture base sequence.

1.4.2.4 Linkage

Alternatively, a target detection site can be directly linked to a solid support via a reversible or irreversible bond between an appropriate functionality (L') on the target nucleic acid molecule and an appropriate functionality (L) on the capture molecule. A reversible linkage can be such that it is cleaved under the conditions of mass spectrometry (i.e., a photocleavable bond such as a trityl ether bond or a charge transfer complex or a labile bond being formed between relatively stable organic radicals). Furthermore, the linkage can be formed with L' being a quaternary ammonium group, in which case, preferably, the surface of the solid support carries negative charges which repel the negatively charged nucleic acid backbone and thus facilitate the desorption required for analysis by a mass spectrometer. Desorption can occur either by the heat created by the laser pulse and/or, depending on L', by specific absorption of laser energy which is in resonance with the L' chromophore.

By way of example, the L-L' chemistry can be of a type of disulfide bond (chemically cleavable, for example, by mercaptoethanol or dithioerythrol), a biotin/streptavidin system, a heterobifunctional derivative of a trityl ether group (Köster et al., "A Versatile Acid-Labile Linker for Modification of Synthetic Biomolecules," Tetrahedron Letters 31, 7095 (1990)) which can be cleaved under mildly acidic conditions as well as under conditions of mass spectrometry, a levulinyl group cleavable under almost neutral conditions with a hydrazinium/acetate buffer, an arginine-arginine or lysine-lysine bond cleavable by an endopeptidase enzyme like trypsin or a pyrophosphate bond cleavable by a pyrophosphatase or a ribonucleotide in between a deoxynucleotide sequence cleavable by an RNase or alkali.

The functionalities, L and L', can also form a charge transfer complex and thereby form the temporary L-L' linkage. Since in many cases the "charge-transfer band" can be determined by UV/vis spectrometry (see e.g. Organic Charge Transfer Complexes by R. Foster, Academic Press, 1969), the laser energy can be tuned to the corresponding energy of the charge-transfer wavelength and, thus, a specific desorption off the solid support can be initiated. Those skilled in the art will recognize that several combinations can serve this purpose and that the donor functionality can be either on the solid support or coupled to the nucleic acid molecule to be detected or vice versa.

In yet another approach, a reversible L-L' linkage can be generated by homolytically forming relatively stable radicals. Under the influence of the laser pulse, desorption (as discussed above) as well as ionization will take place at the radical position. Those skilled in the art will recognize that other organic radicals can be selected and that, in relation to the dissociation energies needed to homolytically cleave the bond between them, a corresponding laser wavelength can be selected (see e.g. Reactive Molecules by C. Wentrup, John Wiley & Sons, 1984). An anchoring function L' can also be incorporated into a target capturing sequence by using appropriate primers during an amplification procedure, such as PCR, LCR or transcription amplification.

For certain applications, it may be useful to simultaneously amplify and chain terminate more than one (mutated) loci on a particular captured nucleic acid fragment (on one spot of an array) or it may be useful to perform parallel processing by using oligonucleotide or oligonucleotide mimetic arrays on various solid supports.

"Multiplexing" can be achieved either by the sequence itself (composition or length) or by the introduction of mass-modifying functionalities into the primer oligonucleotide. Such multiplexing is particularly useful in conjunction with mass spectrometric DNA sequencing or mobility modified gel based fluorescence sequencing.

1.4.2.5 Mass or Mobility Modification

Without limiting the scope of the invention, the mass or mobility modification can be introduced by using oligo/polyethylene glycol derivatives. The oligo/polyethylene glycols can also be monoalkylated by a lower alkyl such as methyl, ethyl, propyl, isopropyl, t-butyl and the like. Other chemistries can be used in

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After PAGE, the gels are exposed to X-ray films and silver grain exposure is analyzed.

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To demonstrate the sequence specificity of target hybridization, two different probes were synthesized in 800 x 1280 μm stripes. The probe S-3'-CGCATCCG was synthesized in stripes 1, 3 and 5. The probe S-3'-CGCTTCCG was synthesized in stripes 2, 4 and 6. The results of hybridizing a 5'-GCGTAGGC-fluorescein target to the substrate at 15°C are depicted herein. Although the probes differ by only one internal base, the target hybridizes specifically to its

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In a light-directed synthesis, the location and composition of products depends on the pattern of illumination and the order of chemical coupling reagents (see Fodor et al., Science (1991) 251:767-773, for a complete description). Consider the synthesis of 256 tetranucleotides. Mask 1 activates one fourth of the substrate surface for coupling with the first of four nucleosides in the first round of synthesis. In cycle 2, mask 2 activates a different quarter of the substrate for coupling with the second nucleoside. The process is continued to build four regions of mononucleotides. The masks of round 2 are perpendicular to those of round 1, and each cycle of round 2 generates four new dinucleotides. The process continues through round 2 to form sixteen dinucleotides. The masks of round 3 further subdivide the synthesis regions so that each coupling cycle generates 16 trimers. The subdivision of the substrate is continued through round 4 to form the tetranucleotides. The synthesis of this probe matrix can be compactly represented in polynomial notation as $(A+C+G+T)^4$. Expansion of this polynomial yields the 256 tetranucleotides.

1.4.3.3 Mismatch Analysis.

1.4.3.3.1 Arrays Used to Determine the Gene Sequence of Oligos of Length "n" Using Array of Probes of Shorter Length "k"

The arrays discussed herein can be utilized in the present method to determine the nucleic acid sequence of an oligonucleotide of length n using an array of probes of shorter length k. In a simple example, the target has a sequence 5'-XXYXY-3', where X and Y are complementary nucleic acids such as A and T or C and G. For discussion purposes, the example is simplified by using only two bases and very short sequences, but the technique can easily be extended to larger nucleic acids with, for example, all 4 RNA or DNA bases.

The sequence of the target is, generally, not known ab initio. One can determine the sequence of the target using the present method with an array of shorter probes. In this example, an array of all possible X and Y 4-mers is synthesized and then used to determine the sequence of a 5-mer target.

Initially, a "core" probe is identified. The core probe is exactly complementary to a sequence in the target using the mismatch analysis method of the present invention. The core probe is identified using one or both of the following criteria:

1. The core probe exhibits stronger binding affinity to the target than other probes, typically the strongest binding affinity of any probe in the array (that has not been identified as a core probe in a previous cycle of analysis).
2. Probes that are mismatched with the target, as compared to the core probe sequence, exhibit a characteristic pattern, discussed in greater detail below, in which probes that mismatch at the 3'- and 5'-end of the probe bind more strongly to the target than probes that mismatch at interior positions.

In this particular example, selection criteria #1 identifies a core 4-mer probe with the strongest binding affinity to the target that has the sequence 3'-YYXY. The probe 3'-YYXY (corresponding to the 5'-XXYX position of the target) is, therefore, chosen as the "core" probe.

Selection criteria #2 is utilized as a "check" to ensure the core probe is exactly complementary to the target nucleic acid.

The second selection criteria evaluates hybridization data (such as the fluorescence intensity of a labeled target hybridized to an array of probes on a

with the same name and the same initials. The first time I had a chance to see the man, I was surprised to find that he was a very young man, only about thirty years of age. He was a very handsome man, with dark hair and a very pleasant smile. He was wearing a dark suit and a white shirt with a dark tie. He was standing in the middle of the room, looking at me with a friendly expression. I was standing in the doorway, looking at him with a surprised expression. He was the same man who had been with me in the car. I was very surprised to see him again. He was the same man who had been with me in the car. I was very surprised to see him again.

An illustrative, hypothetical plot of expected binding affinity versus mismatch position is provided herein. The binding affinity values (typically fluorescence intensity of labeled target hybridized to probe, although many other factors relating to affinity may be utilized) are all normalized to the binding affinity of S-3'-YYXY to the target, which is plotted as a value of 1. Because only two nucleotides are involved in this example, the value plotted for a probe mismatched at position 1 (the nucleotide at the 3'-end of the probe) is the normalized binding affinity of S-3'-XYXY. The value plotted for mismatch at position 2 is the normalized affinity of S-3'-YXXY. The value plotted for mismatch at position 3 is the normalized affinity of S-3'-YYYY, and the value plotted for mismatch position 4 is the normalized affinity of S-3'-YYXX. As noted above, "affinity" may be measured in a number of ways including, for example, the number of photon counts from fluorescence markers on the target.

Of course, in certain situations, as noted in in the section above, identification of a core is all that is required such as in, for example, forensic or genetic studies, and the like.

In sequencing studies, this process is then repeated for left and/or right extensions of the core probe. In one example, only right extensions of the core probe are possible. The possible 4-mer extension probes of the core probe are 3'-YXYY and 31-YXYX. Again, the same selection criteria are utilized. Between 31-YXYY and 3'-YXYX, it would normally be found that 31-YXYX would have the strongest binding affinity, and this probe is selected as the correct probe extension. This selection may be confirmed by again plotting the normalized binding affinity of probes with single base mismatches as compared to the core probe.

When a hypothetical plot is illustrated, again, the characteristic "smile" pattern is observed, indicating that the "correct" extension has been selected, i.e., 3'-YXYX. From this information, one would correctly conclude that the sequence of the target is 51-XXYXY.

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and representative amplification of human genomic DNA sequences from complex sources," *Genomics*, vol. 19, no. 3, pp. 506-14, 1994), incorporated by reference.

When the clonal inserts are sufficiently large to contain inter-Alu regions, and the vector genome is not complex (e.g., bacterial), then IRE-bubble PCR is the preferred embodiment. This situation applies to many clone libraries, including cosmids, PACs, BACs, and P1s.

When the clonal inserts are too small to contain inter-Alu subsequences detectable by hybridization (such as cDNAs), an assay that provides for more uniform DNA expression from the long-range probes may be needed. The most preferred embodiment is then to use a multiplicity of restriction enzyme digests, each followed by long PCR between Alu repeats, and to then pool the PCR products to construct a probe. A second approach is a variation on direct selection (M. Lovett, J. Kere, and L. M. Hinton, "Direct selection: a method for the isolation of cDNAs encoded by large genomic regions," *Proc. Natl. Acad. Sci. U.S.A.*, vol. 88, pp. 9628-9632, 1991), incorporated by reference. In this approach, Lovett's cDNAs are replaced by a full restriction digest with a frequent-cutter of the long-range probe DNA, and Lovett's genomic contig is replaced with repetitive DNA (e.g., Alu or Cot-1) that selects for the same genome as the species-specific long-range probe. The result is a PCR amplification (via the end priming sites) of the long-range probe that is species specific (via the Alu selection).

The species-specific DNA is then amplified and labeled for use as a hybridization probe. In the preferred embodiment, this amplification and labeling is performed using a labeled dNTP with the random primer method (A. P. Feinberg and B. Vogelstein, "A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity," *Analyt. Biochem.*, vol. 132, pp. 6-13, 1983; N.J. Dracopoli, J. L. Haines, B. R. Korf, C. C. Morton, C. E. Seidman, J. G. Seidman, D. T. Moir, and D. Smith, ed., *Current Protocols in Human Genetics*. New York: John Wiley and Sons, 1995), incorporated by reference. In one embodiment, ^{32}P - dNTP is incorporated into a random primer PCR amplification, possibly using a kit such as the DECprime II DNA labeling kit (Ambion, Austin, Tex.). Other isotopes such as ^{35}S or ^{33}P can be used. In alternative embodiments, nonisotopic labeling is performed (L. J. Kricka, ed., *Nonisotopic Probing, Blotting, and Sequencing*, Second Edition. San Diego, Calif.: Academic Press, 1995), incorporated by reference.

1.4.4.1.4 Comparing DNA from the clone library with DNA from the long-range probe library.

The labeled long-range probe DNA is hybridized against the gridded clone library (A. P. Monaco, V. M. S. Lam, G. Zehetner, G. G. Lennon, C. Douglas, D. Nizetic, P. N. Goodfellow, and H. Lehrach, "Mapping irradiation hybrids to cosmid and yeast artificial chromosome libraries by direct hybridization of Alu-PCR products," Nucleic Acids Res., vol. 19, no. 12, pp. 3315-3318, 1991), incorporated by reference. In an alternative embodiment, the roles of the long-range probe library and the clone library are reversed, with the long-range probe immobilized on the membrane and the label on the clone.

The hybridization comparison is done by preannealing the probe with 25 ng of Cot-1 DNA (Gibco-BRL, Grand Island, N.Y.) for 2 hours at 37°C. before adding to the prehybridization mix. The nylon filters containing the spotted clone DNA is then prehybridized overnight per manufacturer's instructions (Amersham, Arlington Heights, Ill.), except for the addition of sheared, denatured human placental DNA at a final concentration of 50 ng/ml. Filters are hybridized overnight at 68°C., washed three times with final wash of 0.1 SSPE/0.1% SDS at 72° C., before exposing to autoradiographic film for 1 to 8 days. The exposed film image is then electronically scanned into a computer with memory. A phosphorimager (Molecular Dynamics, Sunnyvale, Calif.) or other electronic device can be used for imaging without the use of film.

For every RH hybridization probing, each of the clone positions on the autoradiographs of the gridded filters are scored on a numerical scale, such as 1-5, with 1 negative, 2 equivocal., 3 weakly positive, 4 positive, and 5 strongly positive. When duplicate typings are available, the maximum of the two scores is used, since there is a very high false-negative rate in the hybridization data. This data entry can be facilitated by use of an interactive computer program that presents the electronic image of the filter on a computer display, or by automated computer interpretation of the scanned image.

1.4.4.2 Producing a clone library characterized by long-range probes.

The hybridization experiments construct a table of scores that compare the DNA from clones against DNA from long-range probes for detectable sequence similarity, and thus presumed genomic colocalization. The scores are rescaled so that

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1.4.4.2.2 Comparing DNA from the bin probe library with DNA from the long-range probe library.

The orders of the STSs relative to the genome are then determined using computational or statistical methods (M. Boehnke, "Radiation hybrid mapping by minimization of the number of obligate chromosome breaks," Genetic Analysis Workshop 7: Issues in Gene Mapping and the Detection of Major Genes. Cytogenet Cell Genet, vol. 59, pp. 96- 98, 1992; M. Boehnke, K. Lange, and D. R. Cox, "Statistical methods for multipoint radiation hybrid mapping," Am. J. Hum. Genet., vol. 49, pp. 1174-1188, 1991; A. Chakravarti and J. E. Reefer, "A theory for radiation hybrid (Goss-Harris) mapping: application to proximal 21q markers," Genetic Analysis Workshop 7: Issues in Gene Mapping and the Detection of Major Genes. Cytogenet Cell Genet, vol. 59, pp. 99-101, 1992), incorporated by reference. Physical distances are then computed using maximum likelihood estimation.

In the first alternative FISH embodiment of step 7, DNA from the long-range probes (e.g., species-specific PCR products) are fluorescently labeled, and then hybridized back onto the genome. The fragment positions on the genome of the probes are then visualized using fluorescent microscopic imaging. Linear fractional length measurements on the metaphase spreads of chromosomes are then performed

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to determine the bin positions of the fragments. In the second alternative embodiment of step 7, DNA from the previously positioned bin probes is hybridized to DNA from the long-range probes.

Detailed protocols for these methods have been described (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, ed., Current Protocols in Molecular Biology. New York, N.Y.: John Wiley and Sons, 1995; N. J. Dracopoli, J. L. Haines, B. R. Korf, C. C. Morton, C. E. Seidman, J. G. Seidman, D. T. Moir, and D. Smith, ed., Current Protocols in Human Genetics. New York: John Wiley and Sons, 1995), incorporated by reference.

1.4.4.2.3. Producing a long-range probe library whose DNA sequences have been characterized by binning information relative to the genome.

The procedures produce a data table which compares the DNA content of the long-range probes to bins on the genome. In the preferred embodiment, this is a table B of long-range probes (the rows of B) vs. ordered STSs (the columns of B). The pairwise distance information between the ordered STSs is also recorded. In alternative embodiments, the table can be arranged similarly.

Knowledge of the genomic positions of the RH fragments enables the desired correction of noisy RH hybridization data, as described next.

1.4.4.3 Producing a binning of the clone library.

The procedures of step 10 produce a table which bins each clone relative to the genome. In the preferred embodiment, this is a table C of clones (the rows of C) vs. ordered bins (the columns of C). Each entry in the table describes the confidence that the clone is located in the bin.

Note that this result C is a binning of clones, not a contig. To form the desired set of mapped overlapping clones, a short-range probing is preferably performed. This probing and contig formation is performed in the following steps.

1.4.4.3.1 Obtaining a short-range probe library relative to the clone library.

Since current clone mapping technology is based on short-range probing, there is a large number of workable approaches. The preferred embodiment uses hybridization assays based on oligonucleotide probes. The design of such experiments has been described (A. J. Cuticchia, J. Arnold, and W. E. Timberlake, "PCAP: probe choice and analysis package, a set of programs to aid in choosing synthetic oligomers

for contig mapping," CABIOS, vol. 9, no. 2, pp. 201-203, 1992; Y.-X. Fu, E. W. Timberlake, and J. Arnold, "On the design of genome mapping experiments using short synthetic oligonucleotides," Biometrics, vol. 48, pp. 337-359, 1992; H. Lehrach, A. Drmanac, J. Hoheisel, Z. Larin, G. Lennon, A. P. Monaco, D. Nizetic, G. Zehetner, and A. Poustka, "Hybridization fingerprinting in genome mapping and sequencing," in Genetic and Physical Mapping I: Genome Analysis, K. E. Davies and S. M. Tilghman, ed. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory, 1990, pp. 39- 81; A. Poustka, T. Pohl, D. P. Barlow, G. Zehetner, A. Craig, F. Michiels, E. Erlich, A.-M. Frischauf, and H. Lehrach, "Molecular approaches to mammalian genetics," in Cold Spring Harbor Symp. Quant. Biol., vol. 51. 1986, pp. 131-139), incorporated by reference.

An efficient design produces 25 to 200 small (preferably 5 bp-15 bp) oligonucleotides which each hybridize, on average, to 5%-95% of the clones. The oligonucleotide sequences are generally designed to preferentially detect sequences that are related to the genes in the genome, rather than to repetitive elements in the genome or to the cloning vector. This selective bias can be achieved either by experimental probings, or by examination of the sequences to be compared. Once designed, these oligonucleotides are preferably ordered from a DNA synthesis service (Research Genetics, Huntsville, Ala.). Alternatively, they can be synthesized on a DNA synthesizer (Applied Biosystems, Foster City, Calif.).

Alternative hybridization embodiments include using clones (or their PCR products) to probe clone libraries, using pools of clones as hybridization probes, and using Southern blotting of digested clones with repetitive element hybridization probes. Enzymatic methods include gel electrophoresis of restriction endonuclease digests of clones, PCR-based STS comparisons, and hybrid methods such as Alu fingerprinting. Other short-range probes can be formed by selective or random retention of fragments produced by genome cutting.

For experimental efficiency, many of these short-range probes work in a multiplexed way, and probe one or more genome regions simultaneously. These probes include oligonucleotides, pooled clones, and repetitive-element fingerprint probes.

1.4.4.3.2 Comparing DNA from the clone library with DNA from the short-range probe library.

This is done by comparison experiments using standard protocols. In the preferred embodiment, DNA from the clones in the clone library is spotted onto nylon membranes. This DNA is comprised of lysed colonies, DNA preps, or species-specific PCR products. The membranes are then prepared for hybridization. Each oligonucleotide short-range probe is then labeled, preferably with ^{32}P using a kinase. The labeled probe is then hybridized to the membranes, followed by rinsing, stringent washing, and autoradiography. The filters may be stripped for subsequent reuse. The autoradiograph spots are then scored on a binary or more continuous (e.g., 0-255) scale.

Specific oligonucleotide hybridization protocols for particular clone libraries and oligonucleotides have been described (A. G. Craig, D. Nizetic, J. D. Hoheisel, G. Zehetner, and H. Lehrach, "Ordering of cosmid clones covering the herpes simplex virus type I," *Nucleic Acids Res.*, vol. 18, no. 9, 2653-60, 1990; R. Drmanac, Z. Strezoska, I. Labat, S. Drmanac, and R. Crkvenjakov, "Reliable hybridization of oligonucleotides as short as six nucleotides," *DNA Cell Biol.*, vol. 9, no. 7, pp. 527-534, 1990; J. D. Hoheisel, G. G. Lennon, G. Zehetner, and J. Lehrach, "Use of high coverage reference libraries of *Drosophila melanogaster* for relational analysis," *J. Mol. Biol.*, vol. 220, pp. 903-914, 1991; F. Michiels, A. G. Craig, G. Zehetner, G. P. Smith, and H. Lehrach, "Molecular approaches to genome analysis: a strategy for the construction of ordered overlapping clone libraries," *CABIOS*, vol. 3, pp. 203-210, 1987; D. Nizetic, R. Drmanac, and J. Lehrach, "An improved bacterial colony lysis procedure enables direct DNA hybridization using short (10, 11 bases) oligonucleotides to cosmids," *Nucleic Acids Res.*, vol. 19, pp. 182, 1991), incorporated by reference.

For alternative short-range probes, the comparison protocols are described (see cited references above).

1.4.4.3.3 Producing a clone library characterized by short-range probes.

The comparison experiments of the previous step construct a table D of scores that compare the DNA from clones against DNA from short-range probes. These provide measures of genomic colocalization and distance.

In this step, or in the following step 15, contigs can be formed from the short-range characterization data of the clones. In the preferred embodiment, each clone's score signature relative to the oligonucleotides is compared against other clones' score

signatures. Pairs of clones having similar score signatures are inferred to be close, and their distances can be estimated. The preferred ordering method is simulated annealing (W. H. Press, B. P. Flannery, S. A. Teukolsky, and W. T. Vetterling, *Numerical Recipes in C: The Art of Scientific Computing*. Cambridge: Cambridge University Press, 1988), incorporated by reference. Effective contigging algorithms have been described (A. J. Cuticchia, J. Arnold, and W. E. Timberlake, "ODS: ordering DNA sequences, a physical mapping algorithm based on simulated annealing," *CABIOS*, vol. 9, no. 2, pp. 215-219, 1992; A. J. Cuticchia, J. Arnold, and W. E. Timberlake, "The Use of Simulated Annealing in Chromosome Reconstruction Experiments Based on Binary Scoring," *Genetics*, vol. 132, pp. 591-601, 1992; A. Milosavljevic, Z. Strezoska, M. Zeremski, D. Grujic, T. Paunesku, and R. Crkvenjakov, "Clone clustering by hybridization," *Genomics*, vol. 27, no. 1, pp. 83-89, 1995), incorporated by reference.

For alternative short-range probes, the contigging analysis procedures use analogous comparison data and search procedures, and have been described (D. O. Nelson and T. P. Speed, "Statistical issues in constructing high resolution physical maps," *Statistical Science*, vol. 9, no. 3, pp. 334-354, 1994; E. Branscomb, T. Slezak, R. Pae, D. Galas, and al., "Optimizing restriction fragment fingerprinting methods for ordering large genomic libraries," *Genomics*, vol. 8, pp. 351-366, 1990; S. G. Fisher, E. Cayanis, J. J. Russo, I. Sunjevaric, B. Boukhgalter, P. Zhang, M.-T. Yu, R. Rothstein, D. Warburton, I. S. Edelman, and A. Efstratiadis, "Assembly of ordered contigs of cosmids selected with YACs of human chromosome 13," *Genomics*, vol. 21, pp. 525-537, 1994; R. Mort, A. Grigoriev, E. Maier, J. Hoheisel, and H. Lehrach, "Algorithms and software tools for ordering clone libraries: application to the mapping of the genome of *Schizosaccharomyces pombe*," *Nucleic Acids Research*, vol. 21, no. 8, pp. 1965-1974, 1993), incorporated by reference.

1.4.4.3.4 Forming a tiling path of clones that span genome regions.

From an accurate clone map of a genome, a (not necessarily unique) subset of clones that cover the genome can be identified. This identification is done by starting from a leftmost clone by moving rightward from a selected clone A, selecting a neighbor B which overlaps A, and then iteratively continuing from B. A constraint

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1.4.5 Insertion of a Genomic Fragment into an Appropriate Host Vector

In another embodiment, the process begins with a fragment of DNA, such as a genomic fragment, which is inserted into an appropriate host vector capable of accommodating it. For example, a BAC vector can accommodate approximately 140 kb of DNA; a cosmid vector can accommodate approximately 40 kb. A composition comprised of these insert-containing vectors is randomly sheared using standard methods, such as sonication, to obtain fragments suitable for transposon-based sequencing--i.e., about 2-5 kb, preferably 3-4 kb, on the average.

The resulting subfragments are ligated into cloning vectors to create a first library of subclones representing the original fragment. Because the subclones in this library will be used as target plasmids for transposon-mediated sequencing, the size of the cloning vector should be minimized; preferably it should contain only a selectable marker, an origin of replication, and an insertion site. A suitable host plasmid is pOT2; the subfragments obtained by shearing the original composition are end-repaired, ligated to suitable restriction site containing adapters, and inserted into the host vector. Suitable adapters for the pOT2 vector contain BstXI sites.

The resulting cloning vectors with their inserts are then transfected into bacteria, typically *E. coli*, for clonal growth. This first library should contain a 15-20-fold representation of the original fragment of DNA. For example, if the original fragment is approximately 40 kb, and the subclones contain inserts of approximately 4 kb, 200 such clones would be required for a 20-fold representation of the original fragment.

1.4.5.1 Hybridization Screening

As pointed out above, this first library will contain subclones which do not contain DNA derived from the original fragment to be sequenced. In order to eliminate these subclones, a preliminary hybridization screen is conducted. The required number of subclones is prepared for hybridization screening, for example, by plating in 96-well plates and transferring to filters. The filters are then probed with the original fragment insert to weed out any colonies which do not contain DNA which represents portions of the original fragment. This checks the quality of the library and eliminates subclones that contain only host cloning vector for the original fragment or contaminating bacterial DNA.

1.4.5.2 2nd Library Formation by Subclones that Contain Inserts

The subclones confirmed to contain inserts derived from the fragment to be sequenced form a second library. The number of subclones in this library should be sufficient to contain a 7-8x times. representation of the fragment. Each subclone is individually sequenced from one end of the insert. This is straightforward, since the sequence information in the cloning vector provides sufficient information to design appropriate primers. Typically, about 400-450 nucleotides into the insert is read. In addition to the requirement for 7-8x times. coverage of the fragment when the complete insert sequences of the subclones are obtained, there must be sufficient sequence information available from this end sequencing to represent a 1x times. coverage of the fragment. Thus, if the original fragment contained 40 kb and 400 nucleotides into the insert is read, 100 clones would be required. The resulting sequence information is organized into a computer-readable form for searching. A DNA sequence comparison algorithm can be used for subsequent comparisons, such as the NCBI program BLASTN.

The criteria used to determine the number of subclones used to establish the database in the method described above are that low sequencing redundancy must be maintained and a complete path must be available within the set of subclones chosen to provide complete coverage of the original fragment. In addition, the number must be chosen so that there is a high probability of finding the next subclone when searching with the newly sequenced end sequence.

A method similar to that employed by Chen, E. et al. Genomics (1993) 17:651-666, is used. Lander and Waterman (cite) conclude that the maximum number of sequence islands occurs at $C=(1-\text{theta.})^{-1}$, where C is the sequence coverage and theta is the ratio of the number of bases required to detect the true overlap to the sequence read length. As theta approaches zero, sequence coverage of 1 will produce the maximum number of sequence islands. In order to achieve the highest efficiency database, enough end-sequence data should be generated to obtain about 1x times. coverage.

In addition, the subclone coverage--i.e., the redundancy based on the complete sequence contained in the number of subclones chosen--is important. A subclone coverage factor of 7x-8x times provides a 99.9% probability that each nucleotide in the fragment will actually reside in the library. This requires only about 100 subclones averaging 3 kb in size for a 40 kb fragment.

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Sequence information from the host vector for the original fragment is used as the first query and reveals which subclones in the library are hybrid vector/fragment insert subclones. These will identify the two ends of the original fragment. One subclone representing each end, preferably that containing the least amount of vector sequence, is selected for further sequencing. The insert of the identified subclone will be sequenced from the opposite end from that previously sequenced-- i.e., opposite the end containing the vector sequence. The new sequence information (which is now derived from the fragment) is used as the next query. This identifies additional subclones which contain additional nucleotide sequence farther in from the end of the original fragment. The next identified subclone is then also sequenced from the opposite end of the insert from that used to place it in the database and the new sequence information used as the next query. The process is continued sequentially until a subclone path through the fragment is obtained. The subclone path will represent the collection of subclones which completely define the fragment from which they originated, and their correct relative positions are known.

At any point in this process, if there are no responses to the query, additional sequence can be obtained from the subclones already identified and this sequence used as the query.

Once the subclone path is determined, it remains only to complete the sequencing of the subclones involved in the path. According to the method of the invention, this is accomplished using the transposon-mediated method of Strathmann incorporated by reference hereinabove. Use of this method to complete the sequence information for the fragment has been designated "minimal assembled path" (MAP) sequencing. The name is apt because the information provided by the subclone path can be used to determine the minimal sequencing path through the identified subclones. For example, if two subclones overlap over 1 kb, transposon insertions can be selected so that the overlap region is sequenced only once. Thus, although theoretically each of the subclones obtained to define the path can be completely sequenced using the transposon-mediated method, only sufficient portions of these subclones need be sequenced to obtain the complete sequence of the original fragment.

1.4.7 Production of the DNA Fragments

In another embodiment, the DNA fragments are preferably prepared according to either the enzymatic or chemical degradation sequencing techniques previously described, but the fragments are not tagged with radioactive tracers. These standard procedures produce, from each section of DNA to be sequenced, four separate collections of DNA fragments, each set containing fragments terminating at only one of the four bases. These four samples, suitably identified, are provided as a few microliters of liquid solution.

1.4.7.1 Sample Preparation and Introduction

To obtain intact molecular ions from large molecules, such as DNA fragments, by UV laser desorption mass spectrometry, the samples should be dispersed in a solid matrix that strongly absorbs light at the laser wavelength. Suitable matrices for this purpose include cinnamic acid derivatives such as (4-hydroxy, 3-methoxy) cinnamic acid (ferulic acid), (3,4-dihydroxy) cinnamic acid (caffeic acid) and (3,5-dimethoxy, 4-hydroxy) cinnamic acid (sinapinic acid). These materials may be dissolved in a suitable solvent such as 3:2 mixture of 0.1% aqueous trifluoroacetic acid and acetonitrile at concentrations which are near saturation at room temperature.

One technique for introducing samples into the vacuum of the mass spectrometer is to deposit each sample and matrix as a liquid solution at specific spots on a disk or other media having a planar surface. To prepare a sample for deposit, approximately 1 microliter of the sample solution is mixed with 5-10 microliters of the matrix solution. An aliquot of this mixed solution for each DNA sample is placed on the disk at a specific location or spot, and the volatile solvents are removed by room temperature evaporation. When the solution containing the samples and thousand-fold or more excess of matrix is dried on the disk, the result should be a solid solution of samples each in the matrix at a specific site on the disk.

Each molecule of the sample should be fully encased in matrix molecules and isolated from other sample molecules. Aggregation of sample molecules should not occur. The matrix need not be volatile, but it must be rapidly vaporized following absorption of photons. This can occur as the result of photochemical conversion to more volatile substances. In addition, the matrix must transfer ionization to the sample. To form protonated positive molecular ions from the sample, the proton affinity of the matrix must be less than that of the basic sites on the molecule, and to

of a dedicated, trained operator to prepare and load the samples, and preferably is automated to produce 50 or more spectrum per hour.

Greater detail of the preferred technique for DNA sequencing is depicted herein. Under the control of the computer, the disk may be rotated by another stepper motor relative to the reference mark to sequentially bring any selected sample to the position for measurement. If the disk contains 120 samples, operator intervention is only required approximately once every two hours to insert a new sample disk, and less than five minutes of each two hour period is required for loading and pumpdown. With this approach, a single operator can service several spectrometers. The particular disk geometry shown for the automated system is chosen for illustrative purposes only. Other geometries, employing for example linear translation of the planar surface, could also be used.

1.4.7.2 The Mass Spectrometer

The present invention preferably utilizes a laser desorption time of flight (TOF) mass spectrometer. The disk has a planar face containing a plurality of sample spots, each being approximately equal to the laser beam diameter. The disk is maintained at a voltage V_1 and may be manually inserted and removed from the spectrometer. Ions are formed by sequentially radiating each spot on the disk with a laser beam from source.

The ions extracted from the face of the disk are attracted and pass through the grid covered holes in the metal plates. The plates are at voltages V_2 and V_3 . Preferably V_3 is at ground, and V_1 and V_2 are varied to set the accelerating electrical potential., which typically is in the range of 15,000-50,000 volts. A suitable voltage $V_1 - V_2$ is 5000 volts and a suitable range of voltages $V_2 - V_3$ is 10,000 to 45,000 volts.

The low mass ions are almost entirely prevented from reaching the detector by the deflection plates. The ions travel as a beam between the deflection plates which suitably are spaced 1 cm. apart and are 3-10 cm long. The first plate is at ground and a second plate receives square wave pulses, for example, at 700 volts with a pulse width in the order of 1 microsecond after the laser strikes the tip. Such pulses suppress the unwanted low mass ions, for example, those under 1,000 Daltons, by deflecting them, so that the low weight ions do not reach the detector, while the higher weight ions pass between the plates after the pulse is off, so they are not deflected, and are detected by detector.

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fragments present in a mixture due to variations in the cleavage chemistry. Because of this variation it is possible for a small peak to go undetected using conventional sequencing techniques. With the present invention, such errors can be immediately detected by noting that the mass differences between detected peaks do not match the apparent sequence. In many cases, the error can be quickly corrected by calculating the apparent mass of the missing base from the observed mass differences across the gap. As a result, the present invention provides sequence data not only much faster than conventional techniques, but also data which is more accurate and reliable. This correction technique will reduce the number of extra runs which are required to establish the validity of the result.



1.4.8 The Amplification Of A DNA Stretch Using The Pcr Procedure With The Knowledge Of Only One Primer

In another embodiment, the present invention enables the amplification of a DNA stretch using the PCR procedure with the knowledge of only one primer. Using this basic method, the present invention describes a procedure by which a very long DNA of the order of millions of nucleotides can be sequenced contiguously, without the need for fragmenting and sub-cloning the DNA. In this method, the general PCR technique is used, but the knowledge of only one primer is sufficient, and the knowledge of the other primer is derived from the statistics of the distributions of oligonucleotide sequences of specified lengths.

1.4.8.1 Method of Sequencing without the Need for Fragmenting or Subcloning

The objects and advantages of the present invention are also achieved by a method comprising:

- a) synthesizing a partly fixed primer, with 4, 5, 6 nucleotide, or longer sequence characters fixed within it. The fixed sequence can be any sequence, with some preferred sequences such as those containing many G-C pairs that increases binding affinity. The fixed position within the primer can be anywhere, with some preferred positions;
- b) taking a very long genomic DNA, either uncloned or a cloned large insert such as the YAC or cosmid in which a short sequence of about 20 characters somewhere within the DNA is known;
- c) synthesizing a primer from the sequence known from the DNA in step b;
- d) radiolabeling the primer in step c;
- e) annealing the primers (from step a, and step d or step g as appropriate) to the DNA in step b, and amplifying the DNA between the attached primers;
- f) performing DNA sequencing of the amplified DNA by the chemical degradation method of Maxam and Gilbert, or carrying out DNA sequencing by the Sanger method, or by modified PCR-sequencing method;
- g) after obtaining the DNA sequence from step f, selecting an appropriate first primer towards the 3' end of the sequence, synthesizing it, and radiolabeling it;

1. The first part of the document discusses the importance of maintaining accurate records of all transactions and activities. It emphasizes the need for transparency and accountability in financial reporting.

2. The second part of the document outlines the various methods and techniques used to collect and analyze data. It includes a detailed description of the experimental setup and the procedures followed during the study.

3. The third part of the document presents the results of the study, including a comparison of the findings with previous research. It highlights the key observations and conclusions drawn from the data analysis.

4. The fourth part of the document discusses the implications of the study and provides recommendations for future research. It also addresses the limitations of the study and suggests ways to improve the methodology.

5. The final part of the document is a conclusion that summarizes the main findings and reiterates the significance of the study. It also includes a list of references and a list of figures and tables.

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Said ordered arrays have been formed on the surfaces of integrated electronic devices. It has been shown that, provided stringency can be made sufficiently high to prevent binding with even one base mismatch, such methods may be used to obtain sequence information about a sufficiently small sample.

The methods of the present invention provide a more rapid and convenient method for testing for the binding of known oligonucleotides to a complex polynucleotide sample, owing largely to the higher degree of parallelism which may be accomplished with single molecule methods. Here, each oligonucleotide, of known sequence, to be used as a specific gene probe, is synthesized with some perceptible encoded label, as described above, where the codes assigned to the sequence of said each oligonucleotide are known (due to the synthetic scheme by which they are produced and concurrently labeled). These are then hybridized to sample polynucleotide molecules, which either have previously been or will subsequently be immobilized, or may otherwise be separated from probe oligonucleotides, and the presence or absence of said each oligonucleotide in the sample polynucleotide containing fraction, which is a direct result of the success or failure of said each oligonucleotide to bind said sample polynucleotide molecules, will be readily ascertained through the detection and discrimination of the perceptible encoding labels corresponding to said each oligonucleotide. Contrary to the conventional gene-probe methodology, known probing molecules are generally unbound in this variation of the method as may be used with the present invention.

If the complexity of the polynucleotide sample is not too large, and the population made up of said oligonucleotides is sufficiently large and complex, preferably exhaustively enumerating all possible oligonucleotides of the respective and sufficiently long length, and provided hybridization may be sufficiently stringent, which stringency is affected by a large number of known factors but also has sequence dependent components, information about the binding of said each oligonucleotide, which may be related to the respective known sequence and by Watson-Crick pairing rules to the respective sample polynucleotide sequence segment (or by identity with the strand complementary to the strand to which said each oligonucleotide has bound) may thus be obtained. As with other methods, alignment of such data may yield information about the sequence of the sample. The methods of the present invention further provide for the quantitation of such oligonucleotide

hybridization by way of counting the number of times a particular perceptible encoded label is retained by a said polynucleotide sample, which may be availed both in the monitoring and correcting of errors and in the modulation of binding (hybridization) conditions.

Alternatively, probing may be accomplished by oligomeric sequences immobilized in some known configuration, for example by spatially patterned methods such as those of S.P.A. Fodor et al.³⁷ or by the lattices produced hierarchically by the method of N.C. Seeman noted above but comprising an ordered array (the order of which is predetermined by the incorporation or association of single stranded oligonucleotides or other single stranded termini of known sequence into or with modular components used to build up said lattices) of short single stranded regions of known sequence and preferably one free terminus (so as not to hinder conformational changes required for hybridization), but detected by the methods of the present invention, where sample polynucleotides are labeled with some appropriate discernible label, such as the dye YOYO-I, to facilitate the detection of their presence in association with each of said oligomeric sequences.

A yet further variation for effecting the spatially predetermined distribution of, for example and exhaustively enumerated population of single stranded oligonucleotides, may be effected by the used of the methods of N.C. Seeman to produce a uniform two dimensional lattice with a repeating pattern of short single stranded sequences with photo protected termini, for example all of the 256 possible 4-mers. Such a lattice may have a periodicity substantially smaller than the wavelength of visible light. Said short single stranded sequences may be comprise some synthetic backbone so as to be resistant to enzymatic cleavage, which backbone preferably also is non-ionic (for example, of alkyl or beta-cyanoethyl derivation, peptide-nucleic-acid composition, or methylphosphonate composition) so as to denature from a complementary sequence only at markedly elevated temperatures relative to ordinary oligonucleotides. Thus, a pattern of oligonucleotide complexity may be distributed in a predetermined manner below the resolution of light directed patterning.

Light patterning techniques may then be availed to spatially direct the photodeprotection of said short single stranded sequences at lower resolution. Such light directed syntheses are preferably terminated with some comonomer which will

prevent exonucleolytic degradation of said short single stranded sequences, or all of said short single stranded sequences are of a polarity opposite to that specified by the exonuclease to be subsequently used. By this combination of methods, patterning resolution is not limited by the properties of light, but may avail of the convenience of light directed patterning at lower resolutions. After a known distribution of all possible single stranded sequences of sufficient complexity has thus been produced, a denatured, labeled polynucleotide sample produced by extensive nick translation, with fluorescent labeled nucleotides, of a naturally occurring polynucleotide sample is hybridized to said lattice. Hybridized molecules are treated mildly with a single strand specific nuclease, followed by an exonuclease, to degrade or by the same process to free those regions which are not bound to the probing said short single stranded sequences. Label incorporated into the nick translation products of said polynucleotide sample is then detected and spatially mapped by the methods of the present invention, and binding is thus scored according to the known probing said short single stranded sequences. This method thus avails the molecular parallelism made possible by the molecular recognition, high density and high resolution detection methods availed with the present invention.

Note, finally, that higher density patterning than attainable by conventional light patterning methods may also be effected by scanning probe lithographic methods, such as the use of NFSOM lithography with photodeprotectable groups.

1.4.9.2.1 Methods For Repeatable Detection And Identification Of Single Molecules

Repeatable detection and identification of single molecules is achievable by microscopic labeling with some readily identifiable, e.g. combinatorially or permutationally diverse and readily examined particle or molecule or group of molecules and detection of the thus marked identity of individual free molecules in solution, with removal of excess nucleotides (e.g. by filtration); and, scanning of a liquid sample volume where sample molecules and sample conditions are matched to ensure manageably slow free diffusion of sample molecules permitting tracking of the motions of free individual molecules in solution, as observed by T.T. Perkins et al. for reptation of DNA in solution, in which instance unreacted labeled monomers may be removed, for instance, according to their more rapid diffusion, possibly through a

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error from degrading final data quality. These methods may be applied to either immobilized or unimmobilized sample molecules.

1.4.9.2.1.1 Microscopy Based Detection

Light microscopic visualization represents a particularly convenient and technically simple detection and unique molecule localization method. A visualization method of particular interest for purposes of the present invention in higher performance or more demanding applications is video enhanced confocal fluorescence microscopy (VECFM), preferably utilizing optics well matched to the refractive index of the reaction or detection medium.

As discussed above, various scanning probe microscopies may also be advantageously used within the present invention according to labeling agents and methods used. Most prominent among these are NFSOM and variations thereof, and both contact and non-contact SFM, and variations thereof.

Generally speaking, a microscopy based detection method must be sufficiently convenient, capable of use with a stepper translated or otherwise translatable sample, not destructive of the sample, and capable of detection of any labeling methodology to be used with it. Thus, it is quite likely that many microscopy methodologies not yet developed may readily be employed with the present invention. Further, microscopy and corresponding apparatus shall comprehend any miniaturized or microfabricated microscopy devices or other comparable integrated detection means.

1.4.9.2.1.2 HIGH SENSITIVITY AND SCANNED EXCITATION BEAM FLUORESCENCE CONFOCAL MICROSCOPY

A modification of VECFM which is particularly suited for SMD and SMV relies upon selective fluorescent excitation of an appropriate dye molecule label (or of molecules within a sample with appropriate fluorescent properties independent of labeling) in some sample by means of some tightly defined beam, with dimensions at or near the resolution limit of the apparatus, of an appropriate frequency, or of parametrically controllable frequency, where said beam is caused to scan in a controlled manner through the sample region within the visual field. This microscopy, including numerous variations, may be termed either scanned beam confocal microscopy or steered beam confocal microscopy (in either case, SBCM). Scanning

of said beam through the sample within the visual field may be accomplished by introducing said beam into the optical path of the VECFM via mobile mirrors which may effect said controlled scanning, or by first producing said beam with a pinhole which is itself scanned, before deflection towards the sample via said mirrors, which in the present case may be fixed in position, through the use of pinholes in a rotating disk arranged in one or more spiral arms to effect an approximately rastering illumination of the sample as said disk rotates, or by other means which will be obvious to those skilled in the design of optical instrumentation and microscopy. Said beam will excite fluorescence in any appropriately responsive molecules which occur in its path. An optical splitter may then redirect a fraction of the light transmitted from the sample through the objective lens, and direct it through a narrow bandwidth, high transmissiveness filter, which may be specific for a fixed or for a parametrically controllable variable frequency, to uniquely select the appropriate fluorescent emission frequency, to a highly sensitive photodetector, which may record either intensity as intensity information or as the number of photons detected per unit time, as a function of the region being subjected to fluorescence exiting illumination or being distinctly observed (see below). Thus a high resolution map of the fluorescence of the sample may be reconstructed, and further overlaid images obtained for the same sample and sample location by conventional VECFM means.

Alternatively, the entire sample of visual field may be subjected to illumination by an appropriate excitation frequency, and a pinhole scanned through the portion of the output of said optical splitter, such that light passing through said pinhole will reach said highly sensitive photodetector.

In yet a third, albeit technically more complex implementation, an SLM, may be used in place of said pinhole (in either configuration), and fluorescent excitatory illumination may be either broadly distributed or scanned.

In a fourth, albeit technically more complex implementation, sensitive photodetection may be accomplished with a highly sensitive CCD, and fluorescent excitatory illumination may be either broadly distributed or scanned. At present, CCD sensitivity approaching single photon detection is technically possible though is not practical for high volume applications.

In a fifth implementation, said scanned beam may originate from a laser diode array device or a light emitting diode array device, where only one of, or a contiguous

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1.4.9.2.1.3 REPEATABILITY BY IMMOBILIZATION WITH DISCERNIBLE LOCATION

Surface Immobilization

A large number of methods presently exist to effect the immobilization of macromolecules and other molecules to various surfaces including the, surfaces of optically transparent materials. In general, such methods on the chemical modification of said surfaces such that they will be reactive with or have specific affinity for particular chemical functional groups placed on said macromolecules or molecules.

Applicable methods include those described by S.P.A. Fodor et al effect micropatterned surface immobilization and controlled synthesis polypeptides and polynucleotides, those described by M. Hegner et al.¹⁴ effect the end-wise immobilization of terminally thiol modified double helical DNA molecules to a gold coated surface, or those methods recently used by L. Finzi and J. Gelles¹⁵ to effect end-wise attachment of DNA molecules to an antibody coated glass surface. Many alternative methods will be obvious to those skilled in the relevant arts.

For purposes of genome sequencing applications of the present invention, DNA from a cosmid library which may have been prepared from total genomic material., from a cDNA library derived from a particular tissue type, from a cosmid library which may have been prepared for a single chromosome or group of chromosomes or particular chromosome segments, or directly purified genomic DNA or directly purified RNA from a particular cell type, etc., may be subjected to fragmentation. Physical methods such as shearing with a hypodermic apparatus may be suitable. Where the sample is in the form of duplex DNA, it may be treated with restriction enzymes, which preferably restrict either 6- or 4-base recognition sequences, so as to produce sample molecules of mean length of either 4 kilobases or 256 bases, respectively. Such lengths are sufficiently short to yield a high number density of sample molecules. Said sample molecules may then be appropriately derivatized, for example by fill-in reactions at 5' overhang cohesive termini produced by said restriction enzymes with nucleotides bearing an affinity label or an appropriately reactive chemical functional group.

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For the various applications of the present invention involving the interaction of enzymes with extended linear macromolecules such as polynucleotides, when said extended linear molecules may be conveniently circularized by appropriate treatments (which will generally be obvious to those skilled in the relevant arts), immobilization of said extended linear molecules may be conveniently effected by their concatenation with second extended linear molecules which are likewise conveniently circularized by appropriate treatments (which will again generally be obvious to those skilled in the relevant arts) bearing chemical properties (i.e. functional groups such as thiols or affinity moieties such as biotin) favorable for convenient, specific immobilization to a surface, matrix or other solid support. For purposes of, for example, certain sequencing applications of the present invention, said second extended linear molecules are favorably bound (with methods which will generally be obvious to those skilled in the relevant arts) at a predetermined location along their length, to some protein, which may be an enzyme such as a polymerase, before immobilization. Said second extended linear molecules may have termini with reactive chemical functional groups which may be bound together by the addition of some appropriate reagent such as a chemical cross-linking agent, or with some affinity moiety such as an oligo- or polynucleotide which may be bound together by an appropriately complementary oligonucleotide or polynucleotide (with or without ligation thereof), or some appropriate multifunctional binding protein or receptor. Such an arrangement permits the following steps to be performed: said second extended linear molecule is bound to said enzyme; said protein is caused to bind to said first extended linear molecule (which may be circularized either in a prior or subsequent step); said second extended linear molecule to which said protein has been bound is caused to circularize by appropriate treatment; and if said first extended linear molecule is at this stage

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While the above methods are convenient precisely because they require only the simple optimization of sample molecule density, the resulting random distribution will less than fully utilize available substrate or matrix space and fewer than all sample molecules will be sufficiently well separated for unambiguous resolution of two adjacent sample molecules. Due to the inherent advantages provided by molecular parallelism, this will not in general be a significant constraint. For applications in which a high degree of instrumentation miniaturization is desired, however, a better effective density of usable sample molecules, distributed in either two or three dimensions, may be effected as needed by non-random immobilization methods.

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conditions permitting desired selectivity, specificity or stringency but, where appropriate, preventing polynucleotide cleavage or degradation. Where sequence specific binding is desired, and hierarchically prepared lattices are used, the distribution of particular specificities may be controlled by the staged incorporation of said affinity groups at various hierachial levels of the synthetic procedure. This will permit classification of sequence data according to the location of the sample template molecule from which it is obtained in the lattice (i.e. on the surface or within the matrix). Sequence independent binding of polynucleotides may be effected by the use of proteins such as RecA, histones, Ul, etc.

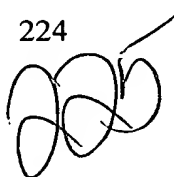
1.4.9.3.1 Repeatable Identification Of Unimmobilized Molecules:

Single molecule tracking with controlled diffusion- For samples under continuous observation, e.g. continuously within as visual field of a video microscope, molecules may be perceptibly labeled, for example by perceptible microscopic beads or the incorporation of a first fluorescent label, and tracked by the use of image analysis algorithms. Said algorithms will recognize only the appropriate type of label and track the motions of the respective sample molecule as it slowly diffuses in solution, so as to permit the unambiguous direct correlation or assignment of the signal associated with the addition of a labeled nucleotide to said respective sample molecule. For these methods, nucleotide labeling does not necessitate the use of large beads or other complexes for detection. Instead, single or oligomeric fluorescent labeling moieties, or enzymatic label affinity conjugation are preferred, such that labels may be removed without greatly disturbing the trajectory of said respective sample molecules. Either the direct colocalization (to within the resolution of the imaging method) of nucleotide label with said first fluorescent label or reductions in the Brownian motion of said nucleotide label sufficiently near (e.g. closest to) said first fluorescent label may be exploited in the detection of nucleotide label incorporation.

Note that manipulation with a laser trap, as for instance described by T.T. Perkins et al. for reptation of DNA in solution, may be employed with such free molecules.

1.4.9.3.2 Unique Labeling Of Sample Molecules And Identification Methods

Various methods may be employed to uniquely label individual sample molecules. The complexity of such unique labels must be greater than the number of



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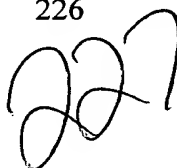
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addition of appropriate adaptor linkers, even in the presence of other biopolymers (such synthetic methods being further favorably facilitated by the use of solid phase synthetic methodologies). Depending on the sensitivity of the detection methods used, multimers of each single type of fluorescent dye moiety, or detectable multiplications of other photolabels, may be used to effect higher modulo coding of labels.

1.4.9.4 ENCODING BY SYNTHESIS WITH MULTIMACROMONOMERS

Note that the labeling methods of the present invention suggest a convenient solution to the problem recognized by Brenner and Lerner, as limiting the facility of their encoding system, i.e. the requirement of separate distinct comonomer (or co-oligomer) type addition steps for each polymer type. This prevents the use of highly random (but step- controlled) synthetic preparation of such encoded libraries, because the information encoded is realized by individual preparative synthetic steps, i.e. all of the information content of the encoding is conferred upon these compounds by the intervention or agency of a chemist (or automated systems) at each step. Such encoded libraries, of either the sequence encoded or modulo encoded types, including compounds comprising more than two polymer types, may be prepared with the following stepped random method in one container (with or without the favorable use of solid phase synthetic methodologies). Note that the term random here refers to the mixture of two or more multimacromonomers in each addition step, such that addition to all compounds under preparation will occur in a random manner within the reaction mixture, in a manner weighted according to the relative concentration of each such multimacromonomer. Such multimacromonomers may also be used in more directly controlled addition schemes with advantages which will be obvious to those skilled in the relevant arts.

Multimacromonomers comprising two or more monomer (or macromonomer) types (e.g. comprising an amino acid monomer and a trinucleotide oligomer, or an amino acid monomer, a trinucleotide oligomer and a fluorescent or affinity labeling moiety) may be prepared by joining some or all of said two or more monomer (or macromonomer) types by cleavable linkers such as those described in other sections of the present disclosure. Thus, each multimacromonomer may be added to compounds under synthesis by addition of one of the monomer or macromonomer types to the corresponding polymer or macropolymer types of said compounds under



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1.4.10 DETECTION METHODS FOR THE PRESENT INVENTION

Non-radioactive labeling techniques have been explored and, in recent years, integrated into partly automated DNA sequencing procedures. These improvements utilize the Sanger sequencing strategy. The label (e.g. fluorescent dye) can be tagged to the primer (Smith et al., Nature M, 674-679 (1986) and EPO Patent No. 87300998.9; Du Pont De Nemours EPO Application No. 0359225; Ansorge et al., J. Biochem. Biophys. Methods 13, 325-32 (1986)) or to the chain-terminating dideoxynucleoside triphosphates (Prober et al. Science 218, 336-41 (1987); Applied Biosystems, PCT Application WO 91/05060). Based on either labeling the primer or the ddNTP, systems have been developed by Applied Biosystems (Smith et al., Science 235, G89 (1987); U. S. Patent Nos. 5 70973 and 689013), Du Pont De Nemours (Prober et al., Science 238, 336-341 (1987); U.S. Patents Nos. 881372 and 57566), Pharmacia-LKB (Ansorge et al., Nucleic Acids Res. 11, 4593-4602 (1987) and EMBL Patent Application DE P3 724442 and P3 805 808. 1) and Hitachi (JP I - 90844 and DE 4011991 AI). A somewhat similar approach was developed by Brumbaugh et al., (Proc. Natl. Acad. Sci. USA 85 5610-14 (1988) and U.S. Patent No. 4,729,947). An improved method for the Du Pont system using two electrophoretic lanes with two different specific labels per lane is described (PCT Application W092/02635). A different approach uses fluorescently labeled avidin and biotin labeled primers. Here, the sequencing ladders ending with biotin are reacted during electrophoresis with the labeled avidin which results in the detection of the individual sequencing bands (Brumbaugh et al., U.S. Patent No. 594676).

More recently even more sensitive non-radioactive labeling techniques for DNA using chemiluminescence triggerable and amplifiable by enzymes have been developed (Beck, O'Keefe, Coull and Köster, Nucleic Acids Res. 12, 5115- 5123 (1989) and Beck and Köster, Anal. Chem. 62, 2258-2270 (1990)). These labeling methods were combined with multiplex DNA sequencing (Church et al., Science 240, 185-188 (1988) and direct blotting electrophoresis (DBE) (Beck and Pohl, EMBO J Vol. 3: p 2905-2909 (1984)) to provide for a strategy aimed at high throughput DNA sequencing (Köster et al., Nucleic Acids Res. Symposium Ser. No. 2,4, 318- 321 (1991), University of Utah, PCT Application No. WO 90/15883). However, this strategy still suffers from the disadvantage of being very laborious and difficult to automate.

Fluorescent labels find use in variety of different biological., chemical., medical and biotechnological applications. One example of where such labels find use is in polynucleotide sequencing, particularly in automated DNA sequencing, which is becoming of critical importance to large scale DNA sequencing projects, such as the Human Genome Project.

In methods of automated DNA sequencing, differently sized fluorescently labeled DNA fragments which terminate at each base in the sequence are enzymatically produced using the DNA to be sequenced as a template. Each group of fragments corresponding to termination at one of the four labeled bases are labeled with the same label. Thus, those fragments terminating in A are labeled with a first label, while those terminating in G, C and T are labeled with second, third and fourth labels respectively. The labeled fragments are then separated by size in an electrophoretic medium and an electropherogram is generated, from which the DNA sequence is determined.

As methods of automated DNA sequencing have become more advanced, of increasing interest is the use of sets of fluorescent labels in which all of the labels are excited at a common wavelength and yet emit one of four different detectable signals, one for each of the four different bases. Such labels provide for a number of advantages, including high fluorescence signals and the ability to electrophoretically separate all of the labeled fragments in a single lane of an electrophoretic medium which avoids problems associated with lane to lane mobility variation.

Although such sets of labels have been developed for use in automated DNA sequencing applications, heretofore the differently labeled members of such sets have each emitted at a different wavelength. Thus, conventional automated detection devices currently employed in methods in which all of the enzymatically produced fragments or primer extension products are separated in the same lane must be able to detect emitted fluorescent light at four different wavelengths. This requirement can prove to be an undesirable limitation. More specifically, carrying out sequencing on vast numbers of different DNA templates simultaneously increases the number of different fragments and corresponding labels required. At the same time, there is a need for a reduction in the complexity of the detection device, e.g. a device which can operate with light detection at only two wavelengths is preferable.

Although the donor and acceptor fluorescer component may be the same, e. g both may be FAM, where they are different the acceptor fluorescer moiety will generally absorb light at a wavelength which is usually at least 10 nm higher, more usually at least 20 nm or higher, than the maximum absorbance wavelength of the donor, and will have a fluorescence emission maximum at a wavelength ranging from about 400 to 900 nm. As with the donor component, the acceptor fluorescer component will have a molecular weight of less than about 2.0 kD, usually less than about 1.5 kD. Acceptor fluorescer moieties may be rhodamines, fluorescein derivatives, BODIPY and cyanine dyes and the like. Specific acceptor fluorescer moieties include FAM, JOE, TAM, ROX, BODIPY and cyanine dyes.

The distance between the donor and acceptor fluorescer components will be chosen to provide for energy transfer from the donor to acceptor fluorescer, where the efficiency of energy transfer will be from 20 to 100 %. Depending on the donor and acceptor fluorescer components, the distance between the two will generally range from 4 to 200 Å, usually from 12 to 100 Å and more usually from 15 to 80 Å, as described above.

For the most part the labels of the subject sets will be described by the following formula:



wherein: D is the donor fluorescer component, which may consist of more than two different donors separated by a spacer;

N is the spacer component, which may be a polymeric chain or rigid chemical moiety, where when N is a polymeric spacer that comprises nucleotides, analogues or mimetics thereof, the number of monomeric units in N will generally range from about 1 to 50, usually from about 4 to 20 and more usually from about 4 to 16;

A is the acceptor fluorescer component, which may consist of more than two different acceptors separated by a spacer; and X is optional and is generally present when the labels are incorporated into oligonucleotide primers, where X is a functionality, e.g an activated phosphate group, for linking to a mono- or

polynucleotide, analogue or mimetic thereof, particularly a deoxyribonucleotide, generally of from 1 to 50, more usually from 1 to 25 nucleotides.

For sets to be employed in nucleic acid enzymatic sequencing in which the labels are to be employed as primers, the labels of the subject sets will comprise either the donor and acceptor fluorescer components attached directly to a hybridizing polymeric backbone, e.g. a polynucleotide, peptide nucleic acid and the like, or the donor and acceptor fluorescer components will be present in an energy transfer cassette attached to a hybridizable component, where the energy transfer cassette comprises the fluorescer components attached to a non-hybridizing polymeric backbone, e.g. a universal spacer. See PCT/US96/13134 and Ju et al., Nat. Med. (1996) supra, the disclosures of which are herein incorporated by reference. The hybridizable component will typically comprise from about 8 to 40, more usually from about 8 to 25 nucleotides, where the hybridizable component will generally be complementary to various commercially available vector sequences such that during use, synthesis proceeds from the vector into the cloned sequence. The vectors may include single-stranded filamentous bacteriophage vectors, the bacteriophage lambda vector, pUC vectors, pGEM vectors, or the like. Conveniently, the primer may be derived from a universal primer, such as pUC/M13, g t10, g t11, and the like, (See Sambrook et al., Molecular Cloning: A Laboratory Manual., 2nd ed., CSHL, 1989, Section 13), where the universal primer will have been modified as described above, e.g. by either directly attaching the donor and acceptor fluorescer components to bases of the primer or by attaching an energy transfer cassette comprising the fluorescer components to the primer.

Sets of preferred energy transfer labels comprising donor and acceptor fluorescers covalently attached to a polynucleotide backbone in the above D-N-A format include: (1) F6R, F 13R, F16R and F16F; where different formats can be employed as long as the four primers display distinct fluorescence emission patterns.

The fluorescent labels of the subject sets can be readily synthesized according to known methods, where the subject labels will generally be synthesized by oligomerizing monomeric units of the polymeric chain of the label, where certain of the monomeric units will be covalently attached to a fluorescer component.

The subject sets of fluorescent labels find use in applications where at least two components of a sample or mixture of components are to be distinguishably

detected. In such applications, the set will be combined with the sample comprising the to be detected components under conditions in which at least two of the components of the sample if present at all will be labeled with first and second labels of the set, where the first and second labels of the set comprise the same donor and acceptor fluorescer components which are separated by different distances. Thus, a first component of the sample is labeled with a first label of the set comprising donor and acceptor fluorescer components separated by a first distance X. A second component of the sample is labeled with a second label comprising the same donor and fluorescer components separated by a second distance Y, where X and Y are as described above. The labeled first and second components, which may or may not have been separated from the remaining components of the sample, are then irradiated by light at a wavelength capable of being absorbed by the donor fluorescer components, generally at a wavelength which is maximally absorbed by the donor fluorescer components. Irradiation of the labeled components results in the generation of distinguishable fluorescence emission patterns from the labeled components, a first fluorescence emission pattern generated by the first label and second pattern being attributable to the second label. The distinguishable fluorescence emission patterns are then detected. Applications in which the subject labels find use include a variety of multicomponent analysis applications in which fluorescent labels are employed, including FISH, micro-array chip based assays where the labels may be used as probes which specifically bind to target components, DNA sequencing where the labels may be present as primers, and the like.

The subject sets of labels find particular use in polynucleotide enzymatic sequencing applications, where four different sets of differently sized polynucleotide fragments terminating at a different base are generated (with the members of each set terminating at the same base) and one wishes to distinguish the sets of fragments from each other. In such applications, the sets will generally comprise four different labels which are capable of acting as primers for enzymatic extension, where at least two of the labels will be energy transfer labels comprising differently spaced common donor and acceptor fluorescer components that are capable of generating distinguishable fluorescence emission patterns upon excitation at a common wavelength of light. Using methods known in the art, a first set of primer extension products all ending in A will be generated by using a first of the labels of the set as a primer. Second, third

and fourth sets of primer extension products terminating in G, C and T will be also be enzymatically produced. The four different sets of primer extension products will then be combined and size separated, usually in an electrophoretic medium. The separated fragments will then be moved relative to a detector (where usually either the fragments or the detector will be stationary). The intensity of emitted light from each labeled fragment as it passes relative to the detector will be plotted as a function of time, i.e. an electropherogram will be produced. Since, the labels of the subject sets will generally emit light in only two wavelengths, the plotted electropherogram will comprise light emitted in two wavelengths. Each peak in the electropherogram will correspond to a particular type of primer extension product (i.e. A, G, C or T), where each peak will comprise one of four different fluorescence emission patterns. To determine the DNA sequence, the electropherogram will be read, with each different fluorescence emission pattern related to one of the four different bases in the DNA chain.

Where desired, two sets of labels according to the subject invention may be employed, where the distinguishable fluorescence emission patterns produced by the labels in the first set will comprise emissions at a first and second wavelength and the patterns produced by the second set of labels will comprise emissions at a third and fourth wavelength. By using two such sets in conjunction with one another, one could detect primer extension products produced from two different template DNA strands at essentially the same time in a conventional four color detector, thereby doubling the throughput of the detector.

The subject sets of labels may be sold in kits, where the kits may or may not comprise additional reagents or components necessary for the particular application in which the label set is to be employed. Thus, for sequencing applications, the subject sets may be sold in a kit which further comprises one or more of the additional requisite sequencing reagents, such as polymerase, nucleotides, dideoxynucleotides and the like.

The following examples are offered by way of illustration and not by way of limitation. The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the subject sets of fluorescent labels.

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1.4.10.2 Affinity labels

Other single molecule detection methods have availed of compounds having well studied affinity interactions with other molecules, such as receptor- ligand interactions.

Genome sequencing applications of the present invention may thus avail of established affinity labeling and detection methods. Other applications of the present invention may also benefit from the application of affinity labeling and detection methods.

Various compounds comprising a nucleotide triphosphate moiety and a small molecule affinity moiety are commercially available and suitable as substrates for DNA polymerases. Said compounds have been used, in conjunction with DNA polymerases, to effect the affinity labeling of various polynucleotide molecules, and thus labeled polynucleotides are routinely subjected to manipulations comprising the formation of an affinity association with an appropriate receptor molecule. Two common examples are the use of biotin as said affinity moiety and streptavidin as said receptor molecule, and digoxigenin as said affinity moiety and anti- digoxigenin antibodies or fragments thereof as the respective said receptor molecule. It will be obvious to those skilled in the relevant arts that there are numerous other possible ligand-receptor interactions which may be exploited for affinity labeling purposes as well as immobilization purposes of the present invention, and that multiple distinct affinity interactions may be employed simultaneously.

For detection purposes, said affinity labels may be used to bind a microscopic colloid or bead which has been modified with an appropriate complementary affinity group such as a receptor.

1.4.10.1 Affinity Label Detection With Microscopic Beads

In recent years a number of different methods and materials have been developed to permit the affinity binding of beads to molecules. Such binding is commonly accomplished by coating said beads with receptor molecules, such as streptavidin or Protein A (also known as Staph A, to which immunoglobulin G antibodies may subsequently be bound). Bead types include polymeric spheres of micron or submicron dimensions, metallic colloids such as colloidal gold, silica beads

above stated limitation, any polymer and respective detection method may be employed.

1.4.10.5 Enzymatic labels and conjugates thereof

1.4.10.5.1 Photochemical labeling

Various methods have been developed for the photochemical labeling of molecules and especially biological macromolecules. These include detection of affinity labels such as biotin with conjugates of streptavidin and an appropriate enzyme capable of catalysing the formation of a chromophore from a chromophorigenic substrate, or capable of catalysing a photon liberating chemical reaction, as with the enzyme luciferase. Such photochemical labeling methods will be readily applicable as detection methods for various embodiments of the present invention.

Note that multimeric affinity labels accessible for simultaneous association with multiple such enzymes will enable greater signal amplification, as will secondary enzyme amplification techniques and other techniques known within the molecular biological and microscopic arts.

1.4.10.5.2 Cleavable linkers

Labeling moieties are favorably in communication with or coupled to nucleotides via a linker of sufficient length to ensure that the presence of said labeling moieties on said nucleotides will not interfere with the action of a polymerase enzyme on said nucleotides. Linkers will also necessarily be of some minimal length when stepping control is effected through the use of various preformed enzyme-nucleotide complexes (as described below). Once a nucleotide has been added by polymerization to (the daughter strand of) a sample molecule, and the accompanying label has been detected, proper detection and discrimination of subsequent nucleotides requires the elimination of said accompanying label. This may favorably be accomplished through the cleavage of said linkers which have been designed and synthesized to admit of cleavage by treatments which will not degrade or otherwise modify the relevant state or information content of sample molecules.

Cleavability may be provided for in a number of ways which will be obvious to those skilled in the arts of organic and synthetic chemistry. For example, said linker may include along its length one or more ester linkages, which will be susceptible to hydrolysis, which may be sufficiently mild for various ester functional

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Note also that for linkers comprising appropriate bonds along their length, enzymatic cleavage may be performed.

Note that cleavage of a labeling moiety may also be effected by the disruption of some affinity interaction which effects the communication between said labeling moiety and the nucleotide moiety. In such cases, moieties joined by non-covalent associations may, for example, be dissociated by physical or chemical changes which do not necessarily cleave covalent bonds.

Similarly, compounds which thermally degrade into two or more portions may comprise an intermediate portion of such linkers, such that thermal cycling may

be employed to effect linker cleavage. Thermostable polymerases may be conveniently employed in embodiments availing thermolabile linkers.

1.4.10.5.4 Photomodification

Single dye molecule photobleaching has been directly observed. Fluorescent labels of nucleotides, particularly when only one or a small number of such moieties are used for labeling, may be neutralized by photobleaching, such that while some product of said fluorescent label may remain in communication with the sample molecule (e.g. the daughter strand of a polynucleotide being sequenced) it will no longer provide a signal sufficiently strong to interfere with the detection and discrimination of subsequently added labels.

Beyond photobleaching of fluorescent labels, affinity labels with appropriate photochemical properties may be subjected to photochemical modification rendering them inert to binding, generally subsequent to dissociation of the corresponding receptor by appropriate means.

For affinity labels, fluorescent labels or any other labeling moieties, chemical modification appropriate to the chemistries of said labels which effects a change or reduction in the detectable signal provided by said label may be availed to prevent interference of said labels with similar or distinct labels subsequently added to sample molecules or complexes thereof.

1.4.10.5.5 Labeling with activation and thermodynamic decay:

Compounds such as spirobenzopyran, which have labile, structurally and photochemically distinct but interconvertible isomers, may be used as labeling moieties. Here, an excited state of such a moiety may be used as a means of detection. After said detection has been successfully effected, chemical modification of one or another state of such interconvertible molecules may then neutralize it. Alternatively, activation may cause such a label to convert to some unstable but discernible state, which then irreversibly degrades according to characterizable kinetics. Such molecules must be chosen so as to remain in said discernible state for a sufficient time period to permit detection, but reliably degrade (to completion for a population of such molecules) within a practical time period.

1.4.10.5.6 Binding reaction inhibition detection methods:

Agents which specifically inhibit binding reactions may be identified rapidly through the detection of molecules, of a diverse library each molecular species of

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Methods are provided to enforce highly specific associations and reactions, including molecular recognition processes, on individual sample molecules or on populations and subpopulations of sample molecules. These are described for genome sequencing applications, but the methods included thereunder have broad applicability, including to any molecular affinity interaction.

Controlled comonomer addition Various methods may be used to accomplish the controlled addition of monomers, including nucleotides and especially labeled or protected nucleotides, to the daughter strand of a sample template molecule.

Means of slowing the time required for the addition of a single nucleotide to a sample molecule will circumvent the requirement of stepping control. This will be particularly applicable for detection mechanisms not requiring separate manipulation steps (such as the separate association of beads to affinity labeled sample molecules). For example, the four nucleotides, each respectively labeled with unique, removable or neutralizable fluorescent labels, may be added to appropriately primed sample template molecules in the presence of polymerases, at low concentrations. Said concentrations must be sufficiently low that two nucleotides are not added to the sample molecule in less than the time required to accomplish the detection of the first such addition. Because all labels are present in the observation field, detection is accomplished through the observation of the reduction of the Brownian motion of a fluorescent moiety due to its addition to the sample molecule, in close analogy to the experiments of Finzi and Gelles, but it will be noted that the change in mobility is much larger in the present case. Alternatively detection may be understood to depend

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on an increase in the net residence of some fluorescent moiety within a defined region or the occupancy of said a region, above the occupancy arising from the background of unbound labeled nucleotides.

Such detection is preferably conducted with a scanning excitation beat fluorescence confocal microscopic method as described above, or with a scanning detection light path, as also described above. Conditions (particularly nucleotide concentration) are chosen such that on average less than one labeled nucleotide will be present within the area illuminated by such a beam or thus observed, so that a light pulse of appropriate frequency passing through, for example, the pinhole which effects the scanning of the excitation beam, may be used to photobleach or photocleave the fluorescent label from the sample molecule after it has been detected to have been added to the sample molecule, without the appreciable accumulation of incidentally unlabeled nucleotides. Alternatively, an SLM may be used to spatially control illumination of the sample by an appropriate frequency of light to effect photochemical unlabeled, and thus permit the simultaneous unlabeled of multiple sample molecules.

This method may be understood as concentration modulated control of the kinetics of polymerization processivity, which is used to facilitate direct observation of successive addition of individual (labeled) nucleotides, with controlled unlabeled. Scanning rate and other instrumentation dependent parameters will influence optimal conditions and concentrations. Thus, direct observation of the addition of comonomers is dynamically observed, and sequence information for the respective sample molecule may be reconstructed accordingly.

1.4.10.7 Stepping control by equilibrium means:

A simple method to effect adequate stepping control for sequencing applications of the present invention relies on equilibrium control. In this method, nucleotides (which are labeled) are limiting, and there is a relative excess of sample molecules. Exonuclease activity intrinsic to most polynucleotide polymerases is circumvented by the use of alpha- phosphorothioate nucleotides (which are appropriately labeled) which are resistant to such degradation, in this method. Other nucleotide derivatives or analogs suitable as substrates for polymerases and yielding exonuclease resistant polynucleotides may likewise be employed.

which will be large for large N . For example, with $x=.03$ and $N=3.3 \times 10^{10}$, there will be a net raw data accumulation of approximately 10^9 bases per cycle, which, with one-hundred-fold oversampling (i.e. due to each sequence being represented 100 times in the sample) will yield 10^7 bases of data per cycle; for a desired segment length of $n=15$ bases, $n/x=(15)/(.03)$ or approximately 500 sequencing cycles will be required per run, and the run will yield 1.5×10^8 bases of information. For polymerase fidelity of 95% (an extremely low value chosen for purposes of illustration) there will be a 5% error rate (e) per base or a segment error rate of $(n)(e)=75\%$ per molecule, but the probability of two erroneous sequence segments having identical sequences will be $e^2(1-e)^{n-1}$ for segments with a single base error, which will be the most frequent error species. For this example, this yields a 0.12% frequency. Methods similar to those used to determine consensus sequences may thus be employed to obtain highly accurate data in spite of less than perfect polymerization fidelity. Thus, fidelity error components will be negligible compared to multiple base incorporation errors. For this example, multiple base incorporation error components will yield an error rate of less than $(2)(15)(.03)^2$ or about 3% per molecule. Again, oversampling will readily detect such errors, which will occur identically for two molecules with only $d^2=(.03)^2$ or less than 0.1% probability, yielding a far lower error rate for over sampled data.

1.4.10.8 Stepping control by removable protecting groups:

Stepping control may favorably be applied to any polymerization process useful within the scope of the present invention, including both genome sequencing and affinity characterization applications.

Template directed polymerization depends on the processive addition of comonomers at the terminus of a growing daughter strand as specified by the respective complementary base of the parent template strand. Complementarity may be enforced through molecular recognition of said complementarity of protected analogs of said comonomers with the appropriate base of a template molecule, by the action upon such protected comonomers of appropriate polymerase enzymes.

Numerous monomers which may thus be added but do not provide an appropriate chemical functional group for subsequent elongation of the polynucleotide strand to which they have been enzymatically added are known within the relevant arts, and are generally referred to as chain terminators. Any such terminators which may be chemically or photochemically modified, particularly in a

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Questions are asked about the following:

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Combinatorial chemistry, affinity characterization, therapeutic synthetic immunochemistry, pharmacology and drug development, in vitro evolution and other

fields concerned with the elaboration of a diverse population of molecules, their characterization according to desired properties, and recovery or identification of molecules displaying suitable characteristics may be favorably improved by the availability of methods which permit the introduction of and both qualitative and quantitative characterization of kinetic and equilibrium properties of molecular recognition and binding phenomena, particularly where such parameters may be used as selective constraints.

There has further been some interest in rebuilding or supplementing the immune systems of immunocompromized individuals, and in the development of highly specific antibiotic agents targeted to antibiotic, antifungal or antiviral resistant or otherwise poorly treatable pathogens. Both of these goals may be furthered by the use of the methods of the present invention as they may readily be applied to the determination of pathogen specificity and antigenicity.

1.4.11.1. Application: Gene finding

An integrated clone map is constructed by the method described herein. When the bin probes include polymorphic genetic markers, and these markers are typed against the DNAs of member of families carrying a genetic trait, that trait can be genetically localized on the map relative to one or more bin probes. Depending on the study design, this genetic localization can be carried out using one of a variety of methods (G. M. Lathrop and J.-M. Lalouel, "Efficient computations in multilocus linkage analysis," Amer. J. Hum. Genet., vol. 42, pp. 498-505, 1988; T. C. Matise, M. W. Perlin, and A. Chakravarti, "Automated construction of genetic linkage maps using an expert system (MultiMap): application to 1268 human microsatellite markers," Nature Genetics, vol. 6, no. 4, pp. 384-390, 1994; E. S. Lander and D. Botstein, "Mapping Complex Genetic Traits in Humans: New Methods Using a Complete RFLP Linkage Map," in Cold Spring Harbor Symposia on Quantitative Biology, vol. LI, Cold Spring Harbor, Cold Spring Harbor Laboratory, 1986, pp. 49-62; L. Penrose, Ann. Eugenics, vol. 18, pp. 120-124, 1953; N. E. Morton, Am. J. Hum. Genet., vol. 35, pp. 201-213, 1983; N. Risch, Am. J. Hum. Genet., vol. 40, pp. 1-14, 1987; E. Lander and D. Botstein, Genetics, vol. 121, pp. 185-199, 1989; N. Risch, "Linkage strategies for genetically complex traits," in three parts, Am. J. Hum. Genet., vol. 46, pp. 222-253, 1990; N. Risch, Genet. Epidemiol., vol. 7, pp. 3-16,

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The method described herein describes an inner product mapping analysis mechanism. Localization profiles are produced that can localize DNA sequences to high resolution. This inner product operation can be applied to somatic cell hybrid deletion panel data, thereby increasing the utility of such data by providing more confident and higher resolution localizations.

Genome mismatch scanning (GMS) (S. F. Nelson, J. H. McCusker, M. A. Sander, Y. Kee, P. Modrich, and P. O. Brown, "Genomic mismatch scanning: a new approach to genetic linkage mapping," *Nature Genetics*, vol. 4, no. May, pp. 11-18, 1993), incorporated by reference, has been described as powerful hybridization-based approach to genetic linkage mapping. GMS has applications both in the mapping of genetic traits and in the diagnosis and prevention of disease. What is currently impeding practical application of the GMS method is the lack of a sequence or map of the human (or animal model) genome that would provide densely spaced (e.g., 1 Mb) hybridization probes for the genome sampling step that scans the mismatched genome DNAs. Applicant's invention discloses a practical method for constructing such a sequence or map of a genome using the method described in the specification. In a preferred embodiment, densely spaced subsequences from the constructed sequence of a genome are used as hybridization probes in GMS. In an alternative embodiment, densely spaced clones (or subsequences therefrom) from the constructed map of a genome are used as hybridization probes in GMS.

A sequence and map of a genome is determined by the method described herein. It is generally believed that such maps can be reliably constructed only from highly reliable and relatively complete data. This belief adds considerably to the time, expense, and effort currently expended in constructing genome maps. However, the method described herein discloses a novel mechanism for constructing highly reliable maps from unreliable and incomplete data (J. von Neumann, "Probabilistic logics and the synthesis of reliable organisms from unreliable components," in Automata

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Studies, C. E. Shannon and J. McCarthy, ed. Princeton, N.J.: Princeton University Press, 1956, pp. 43-98), incorporated by reference.

Specifically:

In step 6, table A's long-range characterization of the clone library can be comprised of very noisy, highly unreliable hybridization data exhibiting large error rates.

In step 9, table B's characterization of the long-range probe library can be sparsely sampled. In some embodiments, a 1 Mb average inter-bin distance suffices for accurate mapping and contig construction.

In step 14, table D's short-range characterization of the clone library has a high tolerance for data errors.

This unobvious result is due to the considerable redundancy in the three data tables, and to the noise filtering and consistency cross-checking capabilities of the analysis methods:

In step 11, table C is a highly reliable binning because the clean PCR-based data table B is used as a global corrective for the noisy complex hybridization-based data table A. This has been empirically demonstrated for human chromosome 11.

In step 16, table E is a highly reliable contiging because every clone has been probed with both long-range and short-range data. Therefore, the global binning information relaxes the requirements on the short-range probings: useful comparisons can be made within a relatively small bin region using imperfect data.

1.4.11.11 Application: Mutation Detection

The techniques described herein will have a wide range of applications, particularly wherever desired to determine if a target nucleic acid has a particular nucleotide sequence or some other sequence differing from a known sequence. For example, one application of the inventions herein is found in mutation detection. These techniques may be applied in a wide variety of fields including diagnostics, forensics, bioanalytics, and others.

For example, assume a "wild-type" nucleic acid has the sequence $5'-N_1N_2N_3N_4$ where, again, N refers to a monomer such as a nucleotide in a nucleic acid and the subscript refers to position number. Assume that a target nucleic acid is to be

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1. The first step is to identify the problem. This involves understanding the current situation and what needs to be changed.

By selection of the single base mismatch probes among all possible probes of a certain length, the number of probes on the substrate can be greatly limited. For example, in a 3-base sequence there are 69 possible DNA base sequences, but there will be only one exact complement to an expected sequence and 9 possible single base mismatch probes. By selecting only these probes, the diversity necessary for screening will be reduced. Preferably, but not necessarily, all of such single base mismatch probes are synthesized on a single substrate. While substrates will often be formed including other probes of interest in addition to the single base mismatches, such substrates will normally still have less than 50% of all the possible probes of n-bases, often less than 30% of all the possible probes of n-bases, often less than 20% of all the possible probes of n-bases, often less than 10% of the possible probes of n-bases, and often less than 5% of the possible probes of n-bases.

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information from the external hit and category information from a relevant hierarchy or hierarchies.

Disclosed is a relational database system for storing biomolecular sequence information in a manner that allows sequences to be catalogued and searched according to association with one or more projects for obtaining full-length biomolecular sequences from shorter sequences. The relational database has sequence records containing information identifying one or more projects to which each of the sequence records belong. Each project groups together one or more biomolecular sequences generated during work to obtain a full-length gene sequence from a shorter sequence. The computer system has a user interface allowing a user to selectively view information regarding one or more projects. The relational database also provides interfaces and methods for accessing and manipulating and analyzing project-based information.

Polymer sequences are assembled into bins. A first number of bins are populated with polymer sequences. The polymer sequences in each bin are assembled into one or more consensus sequences representative of the polymer sequences of the bin. The consensus sequences of the bins are compared to determine relationships, if any, between the consensus sequences of the bins. The bins are modified based on the relationships between the consensus sequences of the bins. The polymer sequences are reassembled in the modified bins to generate one or more modified consensus sequences for each bin representative of the modified bins. In another aspect of the invention, sequence similarities and dissimilarities are analyzed in a set of polymer sequences. Pairwise alignment data is generated for pairs of the polymer sequences. The pairwise alignment data defines regions of similarity between the pairs of polymer sequences with boundaries. Additional boundaries in particular polymer sequences are determined by applying at least one boundary from at least one pairwise alignment for one pair of polymer sequences to at least one other pairwise alignment for another pair of polymer sequences including one of the particular polymer sequences. Additional regions of similarity are generated based on the boundaries.

analyses, database comparisons, and computational algorithms are needed to explore the relationships between sequence and phenotype.

2.2. ANNOTATING – EXEMPLARY ASPECTS

The annotation methods of this invention include those described in PCT patent publication Nos. 98/26407, 98/26408, and 99/49403 and United States Patent Nos. 6,023,659 and 5,953,727 and are herein incorporated by reference in their entirety to the same extent as if each individual patent or patent application were specifically and individually indicated to be incorporated by reference in its entirety.

Thus, in one aspect, this present invention provides relational database systems for storing and analyzing biomolecular sequence information together with biological annotations detailing the source and interpretation the sequence data. The present invention provides a powerful database tool for drug development and other research and development purposes.

The present invention provides relational database systems for storing and analyzing biomolecular sequence information together with biological detailing the source and interpretation the sequence data. Disclosed is a relational database systems for storing and displaying genetic information.

Associated with the database is a software system the allows a user to determine the relative position of a selected gene sequence within a genome. The system allows execution of a method of displaying the genetic locus of a biomolecular sequence. The method involves providing a database including multiple biomolecular sequences, at least some of which represent open reading frames located along a contiguous sequence on an organism's genome. An open reading frame for the sequence is selected and displayed together with adjacent open reading frames located upstream and downstream in the relative positions in which they occur on the contiguous sequence.

The invention provides a method of displaying the genetic locus of a biomolecular sequence. The method involve providing a database including multiple biomolecular sequences, at least some of which represent open reading frames located along a contiguous sequence on an organism's genome. The method further involves identifying a selected open reading frame, and displaying the selected open reading frame together with adjacent open reading frames located upstream and downstream from the selected open reading frame.

The adjacent open reading frames and the selected open reading frame are displayed in the relative positions in which they occur on the contiguous sequence, textually and/or graphically. The method of the invention may be practiced with sequences from microbial organisms, and the sequences may include nucleic acid or protein sequences.

The invention also provides a computer system including a database having multiple biomolecular sequences, at least some of which represent open reading frames located along a contiguous sequence on an organism's genome.

The computer system also includes a user interface capable of identifying a selected open reading frame, and displaying the selected open reading frame together with adjacent open reading frames located upstream and downstream from the selected open reading frame. The adjacent the open reading frames and the selected open reading frame are displayed in the relative positions in which they occur on the contiguous sequence. The user interface may also capable of detecting a scrolling command, and based upon the direction and magnitude of the scrolling command, identifying a new selected open reading frame from the contiguous sequence.

The invention further provides a computer program product comprising a computer-usable medium having computer-readable program code embodied thereon relating to a database including multiple biomolecular sequences, at least some of which represent open reading frames located along a contiguous sequence on an organism's genome. The computer program product includes computer-readable program code for identifying a selected open reading frame, and displaying the selected open reading frame together with adjacent open reading frames located upstream and downstream from the selected open reading frame. The adjacent open reading frames and the selected open reading frame are displayed in the relative positions in which they occur on the contiguous sequence.

Comparative Genomics is a feature of the database system of the present invention which allows a user to compare the sequence data of sets of different organism types. Comparative searches may be formulated in a number of ways using the Comparative Genomics feature. For example, genes common to a set of organisms may be identified through a "commonality" query, and genes unique to one of a set of organisms may be identified through a "subtraction" query.

On the other hand, the fact that the β -phase is not observed in the β -phase region of the β -phase diagram is not surprising. The β -phase is a metastable phase and its formation is kinetically hindered. The β -phase is also a high-temperature phase and its formation is kinetically hindered at low temperatures. The β -phase is also a high-temperature phase and its formation is kinetically hindered at low temperatures.

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A method of using a computer system and a computer program product to present information pertaining to a plurality of sequence records stored in a database are also provided by the present invention. The sequence records contain information identifying one or more projects to which each of the sequence records belong. Each of the projects groups one or more biomolecular sequences generated during work to obtain a full-length gene sequence from a shorter sequence. The method and program involve providing an interface for entering query information relating to one or more projects, locating data corresponding to the entered query information, and displaying the data corresponding to the entered query information.

Additionally, the invention provides a method of using a computer system to present information pertaining to a plurality of sequence records stored in a database. The sequence records contains information identifying one or more projects to which each of the sequence records belong. Each of the projects groups one or more biomolecular sequences generated during work to obtain a full-length gene sequence from a shorter sequence. The method involves displaying a list of one or more project identifiers, determining which project identifier or identifiers from the list is selected by a user, then displaying a second list of one or more biomolecular sequence identifiers associated with the selected project identifier or identifiers, determining which sequence identifier or identifiers from the second list has been selected by a user, and displaying a third list of one or more sequences corresponding to the selected sequence identifier or identifiers. Following the display of the third list, a determination may be made whether and which sequence from the third list has been selected by a user. If a sequence is selected, a sequence alignment search of the selected sequence against other databased sequences may be initiated, and the results of the alignment search displayed.

For Electronic Northern analysis, the invention further provides a computer system including a database having sequence records containing information identifying one or more projects to which each of the sequence records belong, each of said projects grouping one or more biomolecular sequences generated during work to obtain a full-length gene sequence from a shorter sequence. The system also has a user interface capable of allowing a user to select one or more project identifiers or project member identifiers specifying one or more sequences to be compared with one or more cDNA sequence libraries, and displaying matches resulting from that comparison.

Figure 1. The effect of the concentration of the H_2O_2 solution on the amount of the H_2O_2 consumed in the reaction of the H_2O_2 solution with the H_2O_2 solution.

Figure 1. The effect of the concentration of the H_2O_2 solution on the amount of the H_2O_2 consumed in the reaction of the H_2O_2 solution with the H_2O_2 solution.

Figure 1. The effect of the concentration of the H_2O_2 solution on the amount of the H_2O_2 consumed in the reaction of the H_2O_2 solution with the H_2O_2 solution.

Figure 1. The effect of the concentration of the H_2O_2 solution on the amount of the H_2O_2 consumed in the reaction of the H_2O_2 solution with the H_2O_2 solution.

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In another aspect of the invention, sequence similarities and dissimilarities are analyzed in a set of polymer sequences. Pairwise alignment data is generated for pairs of the polymer sequences. The pairwise alignment data defines regions of similarity between the pairs of polymer sequences with boundaries. Additional boundaries in particular polymer sequences are determined by applying at least one boundary from at least one pairwise alignment for one pair of polymer sequences to at least one other pairwise alignment for another pair of polymer sequences including one of the particular polymer sequences. Additional regions of similarity are generated based on the boundaries

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Generally, the present invention provides an improved relational database for storing sequence information. The invention may be employed to investigate data from various sources. For example, it may catalogue animal sequences (e.g., human, primate, rodent, amphibian, insect, etc.), plant sequences, and microbial sequences.

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In vivo shuffling of molecules can be performed utilizing the natural property of cells to recombine multimers. While recombination *in vivo* has provided the major natural route to molecular diversity, genetic recombination remains a relatively complex process that involves 1) the recognition of homologies; 2) strand cleavage, strand invasion, and metabolic steps leading to the production of recombinant chiasma; and finally 3) the resolution of chiasma into discrete recombined molecules. The formation of the chiasma requires the recognition of homologous sequences.

The invention provides a means for generating hybrid polynucleotides which may encode biologically active hybrid polypeptides. In one aspect, the original polynucleotides encode biologically active polypeptides. The method of the invention produces new hybrid polypeptides by utilizing cellular processes which integrate the sequence of the

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For example, gene libraries generated from one or more uncultivated microorganisms are screened for an activity of interest. Potential pathways encoding bioactive molecules of interest are first captured in prokaryotic cells in the form of gene expression libraries. Polynucleotides encoding activities of interest are isolated from such libraries and introduced into a host cell. The host cell is grown under conditions which promote recombination and/or reductive reassortment creating potentially active biomolecules with novel or enhanced activities.

The microorganisms from which the polynucleotide may be prepared include prokaryotic microorganisms, such as Eubacteria and Archaeobacteria, and lower eukaryotic microorganisms such as fungi, some algae and protozoa. Polynucleotides may be isolated from environmental samples in which case the nucleic acid may be recovered without culturing of an organism or recovered from one or more cultured organisms. In one aspect, such microorganisms may be extremophiles, such as hyperthermophiles, psychrophiles, psychrotrophs, halophiles, barophiles and acidophiles. Polynucleotides encoding enzymes isolated from extremophilic microorganisms are particularly preferred. Such enzymes may function at temperatures above 100°C in terrestrial hot springs and deep sea thermal vents, at temperatures below 0°C in arctic waters, in the saturated salt environment of the Dead Sea, at pH values around 0 in coal deposits and geothermal sulfur-rich springs, or at pH

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control and regulate the production of a detectable protein or protein-related array activity from the ligated gene clusters. Use of vectors which have an exceptionally large capacity for exogenous DNA introduction are particularly appropriate for use with such gene clusters and are described by way of example herein to include the f-factor (or fertility factor) of *E. coli*. This f-factor of *E. coli* is a plasmid which affect high-frequency transfer of itself during conjugation and is ideal to achieve and stably propagate large DNA fragments, such as gene clusters from mixed microbial samples. Once ligated into an appropriate vector, two or more vectors containing different polyketide synthase gene clusters can be introduced into a suitable host cell. Regions of partial sequence homology shared by the gene clusters will promote processes which result in sequence reorganization resulting in a hybrid gene cluster. The novel hybrid gene cluster can then be screened for enhanced activities not found in the original gene clusters.

Therefore, in a preferred embodiment, the present invention relates to a method for producing a biologically active hybrid polypeptide and screening such a polypeptide for enhanced activity by:

- 1) introducing at least a first polynucleotide in operable linkage and a second polynucleotide in operable linkage, said at least first polynucleotide and second polynucleotide sharing at least one region of partial sequence homology, into a suitable host cell;
- 2) growing the host cell under conditions which promote sequence reorganization resulting in a hybrid polynucleotide in operable linkage;
- 3) expressing a hybrid polypeptide encoded by the hybrid polynucleotide;
- 4) screening the hybrid polypeptide under conditions which promote identification of enhanced biological activity; and
- 5) isolating the a polynucleotide encoding the hybrid polypeptide.

Methods for screening for various enzyme activities are known to those of skill in the art and discussed throughout the present specification. Such methods may be employed when isolating the polypeptides and polynucleotides of the present invention.

As representative examples of expression vectors which may be used there may be mentioned viral particles, baculovirus, phage, plasmids, phagemids, cosmids, fosmids,

homology, or between quasi-repeated units. The reassortment process recombines and/or reduces the complexity and extent of the repeated sequences, and results in the production of novel molecular species. Various treatments may be applied to enhance the rate of reassortment. These could include treatment with ultra-violet light, or DNA damaging chemicals, and/or the use of host cell lines displaying enhanced levels of "genetic instability". Thus the reassortment process may involve homologous recombination or the natural property of quasi-repeated sequences to direct their own evolution.

Repeated or "quasi-repeated" sequences play a role in genetic instability. In the present invention, "quasi-repeats" are repeats that are not restricted to their original unit structure. Quasi-repeated units can be presented as an array of sequences in a construct; consecutive units of similar sequences. Once ligated, the junctions between the consecutive sequences become essentially invisible and the quasi-repetitive nature of the resulting construct is now continuous at the molecular level. The deletion process the cell performs to reduce the complexity of the resulting construct operates between the quasi-repeated sequences. The quasi-repeated units provide a practically limitless repertoire of templates upon which slippage events can occur. The constructs containing the quasi-repeats thus effectively provide sufficient molecular elasticity that deletion (and potentially insertion) events can occur virtually anywhere within the quasi-repetitive units.

When the quasi-repeated sequences are all ligated in the same orientation, for instance head to tail or vice versa, the cell cannot distinguish individual units. Consequently, the reductive process can occur throughout the sequences. In contrast, when for example, the units are presented head to head, rather than head to tail, the inversion delineates the endpoints of the adjacent unit so that deletion formation will favor the loss of discrete units. Thus, it is preferable with the present method that the sequences are in the same orientation. Random orientation of quasi-repeated sequences will result in the loss of reassortment efficiency, while consistent orientation of the sequences will offer the highest efficiency. However, while having fewer of the contiguous sequences in the same orientation decreases the efficiency, it may still provide sufficient elasticity for the effective recovery of novel molecules. Constructs can be made with the quasi-repeated sequences in the same orientation to allow higher efficiency.

Sequences can be assembled in a head to tail orientation using any of a variety of methods, including the following:

- a) Primers that include a poly-A head and poly-T tail which when made single-stranded would provide orientation can be utilized. This is accomplished by having the first few bases of the primers made from RNA and hence easily removed RNaseH.
- b) Primers that include unique restriction cleavage sites can be utilized. Multiple sites, a battery of unique sequences, and repeated synthesis and ligation steps would be required.
- c) The inner few bases of the primer could be thiolated and an exonuclease used to produce properly tailed molecules.

The recovery of the re-assorted sequences relies on the identification of cloning vectors with a reduced RI. The re-assorted encoding sequences can then be recovered by amplification. The products are re-cloned and expressed. The recovery of cloning vectors with reduced RI can be effected by:

- 1) The use of vectors only stably maintained when the construct is reduced in complexity.
- 2) The physical recovery of shortened vectors by physical procedures. In this case, the cloning vector would be recovered using standard plasmid isolation procedures and size fractionated on either an agarose gel, or column with a low molecular weight cut off utilizing standard procedures.
- 3) The recovery of vectors containing interrupted genes which can be selected when insert size decreases.
- 4) The use of direct selection techniques with an expression vector and the appropriate selection.

Encoding sequences (for example, genes) from related organisms may demonstrate a high degree of homology and encode quite diverse protein products. These types of sequences are particularly useful in the present invention as quasi-repeats. However, while the examples illustrated below demonstrate the reassortment of nearly identical original encoding sequences (quasi-repeats), this process is not limited to such nearly identical repeats.

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$\{ \mathbf{f}_1, \mathbf{f}_2, \mathbf{f}_3, \mathbf{f}_4, \mathbf{f}_5, \mathbf{f}_6, \mathbf{f}_7, \mathbf{f}_8, \mathbf{f}_9, \mathbf{f}_{10}, \mathbf{f}_{11}, \mathbf{f}_{12}, \mathbf{f}_{13}, \mathbf{f}_{14}, \mathbf{f}_{15}, \mathbf{f}_{16}, \mathbf{f}_{17}, \mathbf{f}_{18}, \mathbf{f}_{19}, \mathbf{f}_{20}, \mathbf{f}_{21}, \mathbf{f}_{22}, \mathbf{f}_{23}, \mathbf{f}_{24}, \mathbf{f}_{25}, \mathbf{f}_{26}, \mathbf{f}_{27}, \mathbf{f}_{28}, \mathbf{f}_{29}, \mathbf{f}_{30}, \mathbf{f}_{31}, \mathbf{f}_{32}, \mathbf{f}_{33}, \mathbf{f}_{34}, \mathbf{f}_{35}, \mathbf{f}_{36}, \mathbf{f}_{37}, \mathbf{f}_{38}, \mathbf{f}_{39}, \mathbf{f}_{40}, \mathbf{f}_{41}, \mathbf{f}_{42}, \mathbf{f}_{43}, \mathbf{f}_{44}, \mathbf{f}_{45}, \mathbf{f}_{46}, \mathbf{f}_{47}, \mathbf{f}_{48}, \mathbf{f}_{49}, \mathbf{f}_{50}, \mathbf{f}_{51}, \mathbf{f}_{52}, \mathbf{f}_{53}, \mathbf{f}_{54}, \mathbf{f}_{55}, \mathbf{f}_{56}, \mathbf{f}_{57}, \mathbf{f}_{58}, \mathbf{f}_{59}, \mathbf{f}_{60}, \mathbf{f}_{61}, \mathbf{f}_{62}, \mathbf{f}_{63}, \mathbf{f}_{64}, \mathbf{f}_{65}, \mathbf{f}_{66}, \mathbf{f}_{67}, \mathbf{f}_{68}, \mathbf{f}_{69}, \mathbf{f}_{70}, \mathbf{f}_{71}, \mathbf{f}_{72}, \mathbf{f}_{73}, \mathbf{f}_{74}, \mathbf{f}_{75}, \mathbf{f}_{76}, \mathbf{f}_{77}, \mathbf{f}_{78}, \mathbf{f}_{79}, \mathbf{f}_{80}, \mathbf{f}_{81}, \mathbf{f}_{82}, \mathbf{f}_{83}, \mathbf{f}_{84}, \mathbf{f}_{85}, \mathbf{f}_{86}, \mathbf{f}_{87}, \mathbf{f}_{88}, \mathbf{f}_{89}, \mathbf{f}_{90}, \mathbf{f}_{91}, \mathbf{f}_{92}, \mathbf{f}_{93}, \mathbf{f}_{94}, \mathbf{f}_{95}, \mathbf{f}_{96}, \mathbf{f}_{97}, \mathbf{f}_{98}, \mathbf{f}_{99}, \mathbf{f}_{100} \}$

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In one aspect, one such degenerate oligo (comprised of one degenerate N,N,G/T cassette) is used for subjecting each original codon in a parental polynucleotide template to a full range of codon substitutions. In another aspect, at least two degenerate N,N,G/T cassettes are used – either in the same oligo or not, for subjecting at least two original codons in a parental polynucleotide template to a full range of codon substitutions. Thus, more than one N,N,G/T sequence can be contained in one oligo to introduce amino acid mutations at more than one site. This plurality of N,N,G/T sequences can be directly contiguous, or separated by one or more additional nucleotide sequence(s). In another aspect, oligos serviceable for introducing additions and deletions can be used either alone or in combination with the codons containing an N,N,G/T sequence, to introduce any combination or permutation of amino acid additions, deletions, and/or substitutions.

In another aspect, the present invention provides for the use of degenerate cassettes having less degeneracy than the N,N,G/T sequence. For example, it may be desirable in some instances to use (e.g. in an oligo) a degenerate triplet sequence comprised of only one N, where said N can be in the first second or third position of the triplet. Any other

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totaling from 15 to 100,000) to mutagenesis. Preferably, a separate nucleotide is used for mutagenizing each position or group of positions along a polynucleotide sequence. A group of 3 positions to be mutagenized may be a codon. The mutations are preferably introduced using a mutagenic primer, containing a heterologous cassette, also referred to as a mutagenic cassette. Preferred cassettes can have from 1 to 500 bases. Each nucleotide position in such heterologous cassettes be N, A, C, G, T, A/C, A/G, A/T, C/G, C/T, G/T, C/G/T, A/G/T, A/C/T, A/C/G, or E, where E is any base that is not A, C, G, or T (E can be referred to as a designer oligo). The tables below show exemplary tri-nucleotide cassettes (there are over 3000 possibilities in addition to N,N,G/T and N,N,N and N,N,A/C).

In a general sense, saturation mutagenesis is comprised of mutagenizing a complete set of mutagenic cassettes (wherein each cassette is preferably 1-500 bases in length) in defined polynucleotide sequence to be mutagenized (wherein the sequence to be mutagenized is preferably from 15 to 100,000 bases in length). Thusly, a group of mutations (ranging from 1 to 100 mutations) is introduced into each cassette to be mutagenized. A grouping of mutations to be introduced into one cassette can be different or the same from a second grouping of mutations to be introduced into a second cassette during the application of one round of saturation mutagenesis. Such groupings are exemplified by deletions, additions, groupings of particular codons, and groupings of particular nucleotide cassettes.

Defined sequences to be mutagenized (see Fig. 20) include preferably a whole gene, pathway, cDNA, an entire open reading frame (ORF), and entire promoter, enhancer, repressor/transactivator, origin of replication, intron, operator, or any polynucleotide functional group. Generally, a preferred "defined sequences" for this purpose may be any polynucleotide that a 15 base-polynucleotide sequence, and polynucleotide sequences of lengths between 15 bases and 15,000 bases (this invention specifically names every integer in between). Considerations in choosing groupings of codons include types of amino acids encoded by a degenerate mutagenic cassette.

In a particularly preferred exemplification a grouping of mutations that can be introduced into a mutagenic cassette (see Tables 1-85), this invention specifically provides

for degenerate codon substitutions (using degenerate oligos) that code for 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and 20 amino acids at each position, and a library of polypeptides encoded thereby.

3.2. CHIMERIZATIONS

3.2.1 "SHUFFLING"

Nucleic acid shuffling is a method for *in vitro* or *in vivo* homologous recombination of pools of shorter or smaller polynucleotides to produce a polynucleotide or polynucleotides. Mixtures of related nucleic acid sequences or polynucleotides are subjected to sexual PCR to provide random polynucleotides, and reassembled to yield a library or mixed population of recombinant hybrid nucleic acid molecules or polynucleotides.

In contrast to cassette mutagenesis, only shuffling and error-prone PCR allow one to mutate a pool of sequences blindly (without sequence information other than primers).

The advantage of the mutagenic shuffling of this invention over error-prone PCR alone for repeated selection can best be explained with an example from antibody engineering. Consider DNA shuffling as compared with error-prone PCR (not sexual PCR). The initial library of selected pooled sequences can consist of related sequences of diverse origin (i.e. antibodies from naive mRNA) or can be derived by any type of mutagenesis (including shuffling) of a single antibody gene. A collection of selected complementarity determining regions ("CDRs") is obtained after the first round of affinity selection. In the diagram the thick CDRs confer onto the antibody molecule increased affinity for the antigen. Shuffling allows the free combinatorial association of all of the CDR1s with all of the CDR2s with all of the CDR3s, for example.

This method differs from error-prone PCR, in that it is an inverse chain reaction. In error-prone PCR, the number of polymerase start sites and the number of molecules grows exponentially. However, the sequence of the polymerase start sites and the sequence of the molecules remains essentially the same. In contrast, in nucleic acid

reassembly or shuffling of random polynucleotides the number of start sites and the number (but not size) of the random polynucleotides decreases over time. For polynucleotides derived from whole plasmids the theoretical endpoint is a single, large concatemeric molecule.

Since cross-overs occur at regions of homology, recombination will primarily occur between members of the same sequence family. This discourages combinations of CDRs that are grossly incompatible (e.g., directed against different epitopes of the same antigen). It is contemplated that multiple families of sequences can be shuffled in the same reaction. Further, shuffling generally conserves the relative order, such that, for example, CDR1 will not be found in the position of CDR2.

Rare shufflants will contain a large number of the best (eg. highest affinity) CDRs and these rare shufflants may be selected based on their superior affinity.

CDRs from a pool of 100 different selected antibody sequences can be permuted in up to 1006 different ways. This large number of permutations cannot be represented in a single library of DNA sequences. Accordingly, it is contemplated that multiple cycles of DNA shuffling and selection may be required depending on the length of the sequence and the sequence diversity desired.

Error-prone PCR, in contrast, keeps all the selected CDRs in the same relative sequence, generating a much smaller mutant cloud.

The template polynucleotide which may be used in the methods of this invention may be DNA or RNA. It may be of various lengths depending on the size of the gene or shorter or smaller polynucleotide to be recombined or reassembled. Preferably, the template polynucleotide is from 50 bp to 50 kb. It is contemplated that entire vectors containing the nucleic acid encoding the protein of interest can be used in the methods of this invention, and in fact have been successfully used.

The template polynucleotide may be obtained by amplification using the PCR reaction (USPN 4,683,202 and USPN 4,683,195) or other amplification or cloning

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The number of different specific polynucleotides in the mixture will be at least about 100, preferably at least about 500, and more preferably at least about 1000.

At this step single-stranded or double-stranded polynucleotides, either synthetic or natural, may be added to the random double-stranded shorter or smaller polynucleotides in order to increase the heterogeneity of the mixture of polynucleotides.

It is also contemplated that populations of double-stranded randomly broken polynucleotides may be mixed or combined at this step with the polynucleotides from the sexual PCR process and optionally subjected to one or more additional sexual PCR cycles.

Where insertion of mutations into the template polynucleotide is desired, single-stranded or double-stranded polynucleotides having a region of identity to the template polynucleotide and a region of heterology to the template polynucleotide may be added in a 20 fold excess by weight as compared to the total nucleic acid, more preferably the single-stranded polynucleotides may be added in a 10 fold excess by weight as compared to the total nucleic acid.

Where a mixture of different but related template polynucleotides is desired, populations of polynucleotides from each of the templates may be combined at a ratio of less than about 1:100, more preferably the ratio is less than about 1:40. For example, a backcross of the wild-type polynucleotide with a population of mutated polynucleotide may be desired to eliminate neutral mutations (e.g., mutations yielding an insubstantial alteration in the phenotypic property being selected for). In such an example, the ratio of randomly provided wild-type polynucleotides which may be added to the randomly provided sexual PCR cycle hybrid polynucleotides is approximately 1:1 to about 100:1, and more preferably from 1:1 to 40:1.

The mixed population of random polynucleotides are denatured to form single-stranded polynucleotides and then re-annealed. Only those single-stranded polynucleotides having regions of homology with other single-stranded polynucleotides will re-anneal.

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For example, if a polynucleotide which encodes a protein with increased binding efficiency to a ligand is desired, the proteins expressed by each of the portions of the polynucleotides in the population or library may be tested for their ability to bind to the ligand by methods known in the art (i.e. panning, affinity chromatography). If a polynucleotide which encodes for a protein with increased drug resistance is desired, the proteins expressed by each of the polynucleotides in the population or library may be tested for their ability to confer drug resistance to the host organism. One skilled in the art, given knowledge of the desired protein, could readily test the population to identify polynucleotides which confer the desired properties onto the protein.

It is contemplated that one skilled in the art could use a phage display system in which fragments of the protein are expressed as fusion proteins on the phage surface (Pharmacia, Milwaukee WI). The recombinant DNA molecules are cloned into the phage DNA at a site which results in the transcription of a fusion protein a portion of which is encoded by the recombinant DNA molecule. The phage containing the recombinant nucleic acid molecule undergoes replication and transcription in the cell. The leader sequence of the fusion protein directs the transport of the fusion protein to the tip of the phage particle. Thus the fusion protein which is partially encoded by the recombinant DNA molecule is displayed on the phage particle for detection and selection by the methods described above.

It is further contemplated that a number of cycles of nucleic acid shuffling may be conducted with polynucleotides from a sub-population of the first population, which sub-population contains DNA encoding the desired recombinant protein. In this manner, proteins with even higher binding affinities or enzymatic activity could be achieved.

It is also contemplated that a number of cycles of nucleic acid shuffling may be conducted with a mixture of wild-type polynucleotides and a sub-population of nucleic acid from the first or subsequent rounds of nucleic acid shuffling in order to remove any silent mutations from the sub-population.

hydroxylamine, hydrazine or formic acid. Other agents which are analogues of nucleotide precursors include nitrosoguanidine, 5-bromouracil, 2-aminopurine, or acridine.

Generally, these agents are added to the PCR reaction in place of the nucleotide precursor thereby mutating the sequence. Intercalating agents such as proflavine, acriflavine, quinacrine and the like can also be used. Random mutagenesis of the polynucleotide sequence can also be achieved by irradiation with X-rays or ultraviolet light. Generally, plasmid polynucleotides so mutagenized are introduced into *E. coli* and propagated as a pool or library of hybrid plasmids.

Alternatively the small mixed population of specific nucleic acids may be found in nature in that they may consist of different alleles of the same gene or the same gene from different related species (i.e., cognate genes). Alternatively, they may be related DNA sequences found within one species, for example, the immunoglobulin genes.

Once the mixed population of the specific nucleic acid sequences is generated, the polynucleotides can be used directly or inserted into an appropriate cloning vector, using techniques well-known in the art.

The choice of vector depends on the size of the polynucleotide sequence and the host cell to be employed in the methods of this invention. The templates of this invention may be plasmids, phages, cosmids, phagemids, viruses (e.g., retroviruses, parainfluenzavirus, herpesviruses, reoviruses, paramyxoviruses, and the like), or selected portions thereof (e.g., coat protein, spike glycoprotein, capsid protein). For example, cosmids and phagemids are preferred where the specific nucleic acid sequence to be mutated is larger because these vectors are able to stably propagate large polynucleotides.

If the mixed population of the specific nucleic acid sequence is cloned into a vector it can be clonally amplified by inserting each vector into a host cell and allowing the host cell to amplify the vector. This is referred to as clonal amplification because while the absolute number of nucleic acid sequences increases, the number of hybrids does not increase. Utility can be readily determined by screening expressed polypeptides.

for the removal of neutral mutations that affect unselected characteristics (i.e. immunogenicity). Thus it can be useful to determine which mutations in a protein are involved in the enhanced biological activity and which are not, an advantage which cannot be achieved by error-prone mutagenesis or cassette mutagenesis methods.

Large, functional genes can be assembled correctly from a mixture of small random polynucleotides. This reaction may be of use for the reassembly of genes from the highly fragmented DNA of fossils. In addition random nucleic acid fragments from fossils may be combined with polynucleotides from similar genes from related species.

It is also contemplated that the method of this invention can be used for the *in vitro* amplification of a whole genome from a single cell as is needed for a variety of research and diagnostic applications. DNA amplification by PCR is in practice limited to a length of about 40 kb. Amplification of a whole genome such as that of *E. coli* (5,000 kb) by PCR would require about 250 primers yielding 125 forty kb polynucleotides. This approach is not practical due to the unavailability of sufficient sequence data. On the other hand, random production of polynucleotides of the genome with sexual PCR cycles, followed by gel purification of small polynucleotides will provide a multitude of possible primers. Use of this mix of random small polynucleotides as primers in a PCR reaction alone or with the whole genome as the template should result in an inverse chain reaction with the theoretical endpoint of a single concatamer containing many copies of the genome.

100 fold amplification in the copy number and an average polynucleotide size of greater than 50 kb may be obtained when only random polynucleotides are used. It is thought that the larger concatamer is generated by overlap of many smaller polynucleotides. The quality of specific PCR products obtained using synthetic primers will be indistinguishable from the product obtained from unamplified DNA. It is expected that this approach will be useful for the mapping of genomes.

The polynucleotide to be shuffled can be produced as random or non-random polynucleotides, at the discretion of the practitioner. Moreover, this invention provides a method of shuffling that is applicable to a wide range of polynucleotide sizes and types,

including the step of generating polynucleotide monomers to be used as building blocks in the reassembly of a larger polynucleotide. For example, the building blocks can be fragments of genes or they can be comprised of entire genes or gene pathways, or any combination thereof.

In vivo Shuffling

In an embodiment of *in vivo* shuffling, the mixed population of the specific nucleic acid sequence is introduced into bacterial or eukaryotic cells under conditions such that at least two different nucleic acid sequences are present in each host cell. The polynucleotides can be introduced into the host cells by a variety of different methods. The host cells can be transformed with the smaller polynucleotides using methods known in the art, for example treatment with calcium chloride. If the polynucleotides are inserted into a phage genome, the host cell can be transfected with the recombinant phage genome having the specific nucleic acid sequences. Alternatively, the nucleic acid sequences can be introduced into the host cell using electroporation, transfection, lipofection, biolistics, conjugation, and the like.

In general, in this embodiment, the specific nucleic acids sequences will be present in vectors which are capable of stably replicating the sequence in the host cell. In addition, it is contemplated that the vectors will encode a marker gene such that host cells having the vector can be selected. This ensures that the mutated specific nucleic acid sequence can be recovered after introduction into the host cell. However, it is contemplated that the entire mixed population of the specific nucleic acid sequences need not be present on a vector sequence. Rather only a sufficient number of sequences need be cloned into vectors to ensure that after introduction of the polynucleotides into the host cells each host cell contains one vector having at least one specific nucleic acid sequence present therein. It is also contemplated that rather than having a subset of the population of the specific nucleic acids sequences cloned into vectors, this subset may be already stably integrated into the host cell.

It has been found that when two polynucleotides which have regions of identity are inserted into the host cells homologous recombination occurs between the two

polynucleotides. Such recombination between the two mutated specific nucleic acid sequences will result in the production of double or triple hybrids in some situations.

It has also been found that the frequency of recombination is increased if some of the mutated specific nucleic acid sequences are present on linear nucleic acid molecules. Therefore, in a preferred embodiment, some of the specific nucleic acid sequences are present on linear polynucleotides.

After transformation, the host cell transformants are placed under selection to identify those host cell transformants which contain mutated specific nucleic acid sequences having the qualities desired. For example, if increased resistance to a particular drug is desired then the transformed host cells may be subjected to increased concentrations of the particular drug and those transformants producing mutated proteins able to confer increased drug resistance will be selected. If the enhanced ability of a particular protein to bind to a receptor is desired, then expression of the protein can be induced from the transformants and the resulting protein assayed in a ligand binding assay by methods known in the art to identify that subset of the mutated population which shows enhanced binding to the ligand. Alternatively, the protein can be expressed in another system to ensure proper processing.

Once a subset of the first recombined specific nucleic acid sequences (daughter sequences) having the desired characteristics are identified, they are then subject to a second round of recombination.

In the second cycle of recombination, the recombined specific nucleic acid sequences may be mixed with the original mutated specific nucleic acid sequences (parent sequences) and the cycle repeated as described above. In this way a set of second recombined specific nucleic acids sequences can be identified which have enhanced characteristics or encode for proteins having enhanced properties. This cycle can be repeated a number of times as desired.

It is also contemplated that in the second or subsequent recombination cycle, a backcross can be performed. A molecular backcross can be performed by mixing the

desired specific nucleic acid sequences with a large number of the wild-type sequence, such that at least one wild-type nucleic acid sequence and a mutated nucleic acid sequence are present in the same host cell after transformation. Recombination with the wild-type specific nucleic acid sequence will eliminate those neutral mutations that may affect unselected characteristics such as immunogenicity but not the selected characteristics.

In another embodiment of this invention, it is contemplated that during the first round a subset of the specific nucleic acid sequences can be generated as smaller polynucleotides by slowing or halting their PCR amplification prior to introduction into the host cell. The size of the polynucleotides must be large enough to contain some regions of identity with the other sequences so as to homologously recombine with the other sequences. The size of the polynucleotides will range from 0.03 kb to 100 kb more preferably from 0.2 kb to 10 kb. It is also contemplated that in subsequent rounds, all of the specific nucleic acid sequences other than the sequences selected from the previous round may be utilized to generate PCR polynucleotides prior to introduction into the host cells.

The shorter polynucleotide sequences can be single-stranded or double-stranded. If the sequences were originally single-stranded and have become double-stranded they can be denatured with heat, chemicals or enzymes prior to insertion into the host cell. The reaction conditions suitable for separating the strands of nucleic acid are well known in the art.

The steps of this process can be repeated indefinitely, being limited only by the number of possible hybrids which can be achieved. After a certain number of cycles, all possible hybrids will have been achieved and further cycles are redundant.

In an embodiment the same mutated template nucleic acid is repeatedly recombined and the resulting recombinants selected for the desired characteristic.

Therefore, the initial pool or population of mutated template nucleic acid is cloned into a vector capable of replicating in a bacteria such as *E. coli*. The particular vector is not essential, so long as it is capable of autonomous replication in *E. coli*. In a preferred embodiment, the vector is designed to allow the expression and production of any protein

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The nucleic acid or vector is then introduced into the host cell with a large excess of the wild-type nucleic acid. The nucleic acid of the hybrid and the nucleic acid of the wild-type sequence are allowed to recombine. The resulting recombinants are placed under the same selection as the hybrid nucleic acid. Only those recombinants which retained the desired characteristics will be selected. Any silent mutations which do not provide the desired characteristics will be lost through recombination with the wild-type

desirable that the single polynucleotide strands participate to some extent in annealment with different hybridization partners (i.e. and not merely revert to exclusive reannealment between what were former partners before the denaturation step). The presence of the former hybridization partners in the reaction vessel, however, does not preclude, and may sometimes even favor, reannealment of a single stranded polynucleotide with its former partner, to recreate an original double stranded polynucleotide.

In contrast to this non-enzymatic shuffling step comprised of subjecting double stranded polynucleotide building blocks to denaturation, followed by annealment, the instant invention further provides an exonuclease-based approach requiring no denaturation – rather, the avoidance of denaturing conditions and the maintenance of double stranded polynucleotide substrates in annealed (i.e. non-denatured) state are necessary conditions for the action of exonucleases (e.g., exonuclease III and red alpha gene product). Additionally in contrast, the generation of single stranded polynucleotide sequences capable of hybridizing to other single stranded polynucleotide sequences is the result of covalent cleavage – and hence sequence destruction - in one of the hybridization partners. For example, an exonuclease III enzyme may be used to enzymatically liberate 3' terminal nucleotides in one hybridization strand (to achieve covalent hydrolysis in that polynucleotide strand); and this favors hybridization of the remaining single strand to a new partner (since its former partner was subjected to covalent cleavage).

By way of further illustration, a specific exonuclease, namely exonuclease III is provided herein as an example of a 3' exonuclease; however, other exonucleases may also be used, including enzymes with 5' exonuclease activity and enzymes with 3' exonuclease activity, and including enzymes not yet discovered and enzymes not yet developed. It is particularly appreciated that enzymes can be discovered, optimized (e.g. engineered by directed evolution), or both discovered and optimized specifically for the instantly disclosed approach that have more optimal rates &/or more highly specific activities &/or greater lack of unwanted activities. In fact it is expected that the instant invention may encourage the discovery &/or development of such designer enzymes. In sum, this invention may be practiced with a variety of currently available exonuclease enzymes, as well as enzymes not yet discovered and enzymes not yet developed.

The exonuclease action of exonuclease III requires a working double stranded polynucleotide end that is either blunt or has a 5' overhang, and the exonuclease action is comprised of enzymatically liberating 3' terminal nucleotides, leaving a single stranded 5' end that becomes longer and longer as the exonuclease action proceeds (see Figure 1). Any 5' overhangs produced by this approach may be used to hybridize to another single stranded polynucleotide sequence (which may also be a single stranded polynucleotide or a terminal overhang of a partially double stranded polynucleotide) that shares enough homology to allow hybridization. The ability of these exonuclease III-generated single stranded sequences (e.g. in 5' overhangs) to hybridize to other single stranded sequences allows two or more polynucleotides to be shuffled, assembled, reassembled, &/or concatenated.

Furthermore, it is appreciated that one can protect the end of a double stranded polynucleotide or render it susceptible to a desired enzymatic action of a serviceable exonuclease as necessary. For example, a double stranded polynucleotide end having a 3' overhang is not susceptible to the exonuclease action of exonuclease III. However, it may be rendered susceptible to the exonuclease action of exonuclease III by a variety of means; for example, it may be blunted by treatment with a polymerase, cleaved to provide a blunt end or a 5' overhang, joined (ligated or hybridized) to another double stranded polynucleotide to provide a blunt end or a 5' overhang, hybridized to a single stranded polynucleotide to provide a blunt end or a 5' overhang, or modified by any of a variety of means).

According to one aspect, an exonuclease may be allowed to act on one or on both ends of a linear double stranded polynucleotide and proceed to completion, to near completion, or to partial completion. When the exonuclease action is allowed to go to completion, the result will be that the length of each 5' overhang will extend far towards the middle region of the polynucleotide in the direction of what might be considered a "rendezvous point" (which may be somewhere near the polynucleotide midpoint). Ultimately, this results in the production of single stranded polynucleotides (that can become dissociated) that are each about half the length of the original double stranded polynucleotide (see Figure 1). Alternatively, an exonuclease-mediated reaction can be terminated before proceeding to completion.

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Thus this exonuclease-mediated approach is serviceable for shuffling, assembling &/or reassembling, recombining, and concatenating polynucleotide building blocks, which polynucleotide building blocks can be up to ten bases long or tens of bases long or hundreds of bases long or thousands of bases long or tens of thousands of bases long or hundreds of thousands of bases long or millions of bases long or even longer.

This exonuclease-mediated approach is based on the action of double stranded DNA specific exodeoxyribonuclease activity of *E. coli* exonuclease III. Substrates for exonuclease III may be generated by subjecting a double stranded polynucleotide to fragmentation. Fragmentation may be achieved by mechanical means (e.g., shearing, sonication, etc.), by enzymatic means (e.g. using restriction enzymes), and by any combination thereof. Fragments of a larger polynucleotide may also be generated by polymerase-mediated synthesis.

Exonuclease III is a 28K monomeric enzyme, product of the *xthA* gene of *E. coli* with four known activities: exodeoxyribonuclease (alternatively referred to as exonuclease herein), RNaseH, DNA-3'-phosphatase, and AP endonuclease. The exodeoxyribonuclease activity is specific for double stranded DNA. The mechanism of action is thought to involve enzymatic hydrolysis of DNA from a 3' end progressively towards a 5' direction, with formation of nucleoside 5'-phosphates and a residual single strand. The enzyme does not display efficient hydrolysis of single stranded DNA, single-stranded RNA, or double-stranded RNA; however it degrades RNA in an DNA-RNA hybrid releasing nucleoside 5'-phosphates. The enzyme also releases inorganic phosphate specifically from 3'-phosphomonoester groups on DNA, but not from RNA or short oligonucleotides. Removal of these groups converts the terminus into a primer for DNA polymerase action.

Additional examples of enzymes with exonuclease activity include red-alpha and venom phosphodiesterases. Red alpha (*red* gene product (also referred to as lambda exonuclease) is of bacteriophage origin. The *red* gene is transcribed from the leftward promoter and its product is involved (24 kD) in recombination. Red alpha gene product acts processively from 5'-phosphorylated termini to liberate mononucleotides from duplex

[illegible]

In one aspect, the present invention provides a non-stochastic method termed synthetic ligation reassembly (SLR), that is somewhat related to stochastic shuffling, save that the nucleic acid building blocks are not shuffled or concatenated or chimerized randomly, but rather are assembled non-stochastically.

A particularly glaring difference is that the instant SLR method does not depend on the presence of a high level of homology between polynucleotides to be shuffled. In contrast, prior methods, particularly prior stochastic shuffling methods require that presence of a high level of homology, particularly at coupling sites, between polynucleotides to be shuffled. Accordingly these prior methods favor the regeneration of the original progenitor molecules, and are suboptimal for generating large numbers of novel progeny chimeras, particularly full-length progenies. The instant invention, on the other hand, can be used to non-stochastically generate libraries (or sets) of progeny molecules comprised of over 10^{100} different chimeras. Conceivably, SLR can even be used to generate libraries comprised of over 10^{1000} different progeny chimeras with (no upper limit in sight).

Thus, in one aspect, the present invention provides a method, which method is non-stochastic, of producing a set of finalized chimeric nucleic acid molecules having an overall assembly order that is chosen by design, which method is comprised of the steps of generating by design a plurality of specific nucleic acid building blocks having serviceable mutually compatible ligatable ends, and assembling these nucleic acid building blocks, such that a designed overall assembly order is achieved.

The mutually compatible ligatable ends of the nucleic acid building blocks to be assembled are considered to be “serviceable” for this type of ordered assembly if they enable the building blocks to be coupled in predetermined orders. Thus, in one aspect, the overall assembly order in which the nucleic acid building blocks can be coupled is

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In one exemplification, this invention provides for the chimerization of a family of related genes and their encoded family of related products. In a particular exemplification, the encoded products are enzymes. As a representative list of families of enzymes which may be mutagenized in accordance with the aspects of the present invention, there may be mentioned, the following enzymes and their functions:

1 Lipase/Esterase

- a. Enantioselective hydrolysis of esters (lipids)/ thioesters
 - 1) Resolution of racemic mixtures
 - 2) Synthesis of optically active acids or alcohols from *meso*-diesters
- b. Selective syntheses
 - 1) Regiospecific hydrolysis of carbohydrate esters
 - 2) Selective hydrolysis of cyclic secondary alcohols
- c. Synthesis of optically active esters, lactones, acids, alcohols
 - 1) Transesterification of activated/nonactivated esters
 - 2) Interesterification
 - 3) Optically active lactones from hydroxyesters

- 4) Regio- and enantioselective ring opening of anhydrides
- d. Detergents
- e. Fat/Oil conversion
- f. Cheese ripening

2 **Protease**

- a. Ester/amide synthesis
- b. Peptide synthesis
- c. Resolution of racemic mixtures of amino acid esters
- d. Synthesis of non-natural amino acids
- e. Detergents/protein hydrolysis

3 **Glycosidase/Glycosyl transferase**

- a. Sugar/polymer synthesis
- b. Cleavage of glycosidic linkages to form mono, di-and oligosaccharides
- c. Synthesis of complex oligosaccharides
- d. Glycoside synthesis using UDP-galactosyl transferase
- e. Transglycosylation of disaccharides, glycosyl fluorides, aryl galactosides
- f. Glycosyl transfer in oligosaccharide synthesis
- g. Diastereoselective cleavage of -glucosylsulfoxides
- h. Asymmetric glycosylations
- i. Food processing
- j. Paper processing

4 **Phosphatase/Kinase**

- a. Synthesis/hydrolysis of phosphate esters
 - 1) Regio-, enantioselective phosphorylation
 - 2) Introduction of phosphate esters
 - 3) Synthesize phospholipid precursors
 - 4) Controlled polynucleotide synthesis
- b. Activate biological molecule
- c. Selective phosphate bond formation without protecting groups

100% Satisfaction Guarantee

- b. Hydrolysis of aromatic, heterocyclic, unsaturated aliphatic nitriles to corresponding acids
- c. Hydrolysis of acrylonitrile
- d. Production of aromatic and carboxamides, carboxylic acids (nicotinamide, picolinamide, isonicotinamide)
- e. Regioselective hydrolysis of acrylic dinitrile
- f. -amino acids from -hydroxynitriles

10 Transaminase

- a. Transfer of amino groups into oxo-acids

11 Amidase/Acylase

- a. Hydrolysis of amides, amidines, and other C-N bonds
- b. Non-natural amino acid resolution and synthesis

These exemplifications, while illustrating certain specific aspects of the invention, do not portray the limitations or circumscribe the scope of the disclosed invention.

Thus according to one aspect of this invention, the sequences of a plurality of progenitor nucleic acid templates are aligned in order to select one or more demarcation points, which demarcation points can be located at an area of homology, and are comprised of one or more nucleotides, and which demarcation points are shared by at least two of the progenitor templates. The demarcation points can be used to delineate the boundaries of nucleic acid building blocks to be generated. Thus, the demarcation points identified and selected in the progenitor molecules serve as potential chimerization points in the assembly of the progeny molecules.

Preferably a serviceable demarcation point is an area of homology (comprised of at least one homologous nucleotide base) shared by at least two progenitor templates. More preferably a serviceable demarcation point is an area of homology that is shared by at least half of the progenitor templates. More preferably still a serviceable demarcation point is an area of homology that is shared by at least two thirds of the progenitor templates. Even more preferably a serviceable demarcation points is an area of homology that is shared by



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In a preferred embodiment, this invention provides that the ligation reassembly process is performed exhaustively in order to generate an exhaustive library. In other words, all possible ordered combinations of the nucleic acid building blocks are represented in the set of finalized chimeric nucleic acid molecules. At the same time, in a particularly preferred embodiment, the assembly order (i.e. the order of assembly of each building block in the 5' to 3' sequence of each finalized chimeric nucleic acid) in each combination is by design (or non-stochastic). Because of the non-stochastic nature of this invention, the possibility of unwanted side products is greatly reduced.

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may be a man-made gene pathway. This invention provides that one or more man-made genes generated by this invention may be incorporated into a man-made gene pathway, such as a pathway operable in a eukaryotic organism (including a plant).

It is appreciated that the power of this invention is exceptional, as there is much freedom of choice and control regarding the selection of demarcation points, the size and number of the nucleic acid building blocks, and the size and design of the couplings. It is appreciated, furthermore, that the requirement for intermolecular homology is highly relaxed for the operability of this invention. In fact, demarcation points can even be chosen in areas of little or no intermolecular homology. For example, because of codon wobble, i.e. the degeneracy of codons, nucleotide substitutions can be introduced into nucleic acid building blocks without altering the amino acid originally encoded in the corresponding progenitor template. Alternatively, a codon can be altered such that the coding for an originally amino acid is altered. This invention provides that such substitutions can be introduced into the nucleic acid building block in order to increase the incidence of intermolecularly homologous demarcation points and thus to allow an increased number of couplings to be achieved among the building blocks, which in turn allows a greater number of progeny chimeric molecules to be generated.

In another exemplification, the synthetic nature of the step in which the building blocks are generated allows the design and introduction of nucleotides (e.g. one or more nucleotides, which may be, for example, codons or introns or regulatory sequences) that can later be optionally removed in an in vitro process (e.g. by mutagenesis) or in an in vivo process (e.g. by utilizing the gene splicing ability of a host organism). It is appreciated that in many instances the introduction of these nucleotides may also be desirable for many other reasons in addition to the potential benefit of creating a serviceable demarcation point.

Thus, according to another embodiment, this invention provides that a nucleic acid building block can be used to introduce an intron. Thus, this invention provides that functional introns may be introduced into a man-made gene of this invention. This invention also provides that functional introns may be introduced into a man-made gene pathway of this invention. Accordingly, this invention provides for the generation of a

chimeric polynucleotide that is a man-made gene containing one (or more) artificially introduced intron(s).

Accordingly, this invention also provides for the generation of a chimeric polynucleotide that is a man-made gene pathway containing one (or more) artificially introduced intron(s). Preferably, the artificially introduced intron(s) are functional in one or more host cells for gene splicing much in the way that naturally-occurring introns serve functionally in gene splicing. This invention provides a process of producing man-made intron-containing polynucleotides to be introduced into host organisms for recombination and/or splicing.

The ability to achieve chimerizations, using couplings as described herein, in areas of little or no homology among the progenitor molecules, is particularly useful, and in fact critical, for the assembly of novel gene pathways. This invention thus provides for the generation of novel man-made gene pathways using synthetic ligation reassembly. In a particular aspect, this is achieved by the introduction of regulatory sequences, such as promoters, that are operable in an intended host, to confer operability to a novel gene pathway when it is introduced into the intended host. In a particular exemplification, this invention provides for the generation of novel man-made gene pathways that is operable in a plurality of intended hosts (e.g. in a microbial organism as well as in a plant cell). This can be achieved, for example, by the introduction of a plurality of regulatory sequences, comprised of a regulatory sequence that is operable in a first intended host and a regulatory sequence that is operable in a second intended host. A similar process can be performed to achieve operability of a gene pathway in a third intended host species, etc. The number of intended host species can be each integer from 1 to 10 or alternatively over 10. Alternatively, for example, operability of a gene pathway in a plurality of intended hosts can be achieved by the introduction of a regulatory sequence having intrinsic operability in a plurality of intended hosts.

Thus, according to a particular embodiment, this invention provides that a nucleic acid building block can be used to introduce a regulatory sequence, particularly a regulatory sequence for gene expression. Preferred regulatory sequences include, but are not limited to, those that are man-made, and those found in archeal, bacterial, eukaryotic

(including mitochondrial), viral, and prionic or prion-like organisms. Preferred regulatory sequences include but are not limited to, promoters, operators, and activator binding sites. Thus, this invention provides that functional regulatory sequences may be introduced into a man-made gene of this invention. This invention also provides that functional regulatory sequences may be introduced into a man-made gene pathway of this invention.

Accordingly, this invention provides for the generation of a chimeric polynucleotide that is a man-made gene containing one (or more) artificially introduced regulatory sequence(s). Accordingly, this invention also provides for the generation of a chimeric polynucleotide that is a man-made gene pathway containing one (or more) artificially introduced regulatory sequence(s). Preferably, an artificially introduced regulatory sequence(s) is operatively linked to one or more genes in the man-made polynucleotide, and are functional in one or more host cells.

Preferred bacterial promoters that are serviceable for this invention include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and trp. Serviceable eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Particular plant regulatory sequences include promoters active in directing transcription in plants, either constitutively or stage and/or tissue specific, depending on the use of the plant or parts thereof. These promoters include, but are not limited to promoters showing constitutive expression, such as the 35S promoter of Cauliflower Mosaic Virus (CaMV) (Guilley et al., 1982), those for leaf-specific expression, such as the promoter of the ribulose biphosphate carboxylase small subunit gene (Coruzzi et al., 1984), those for root-specific expression, such as the promoter from the glutamin synthase gene (Tingey et al., 1987), those for seed-specific expression, such as the cruciferin A promoter from *Brassica napus* (Ryan et al., 1989), those for tuber-specific expression, such as the class-I patatin promoter from potato (Rocha-Sasa et al., 1989; Wenzler et al., 1989) or those for fruit-specific expression, such as the polygalacturonase (PG) promoter from tomato (Bird et al., 1988).

Other regulatory sequences that are preferred for this invention include terminator sequences and polyadenylation signals and any such sequence functioning as such in plants, the choice of which is within the level of the skilled artisan. An example of such

sequences is the 3' flanking region of the nopaline synthase (nos) gene of *Agrobacterium tumefaciens* (Bevan, 1984). The regulatory sequences may also include enhancer sequences, such as found in the 35S promoter of CaMV, and mRNA stabilizing sequences such as the leader sequence of Alfalfa Mosaic Virus (AIMV) RNA4 (Brederode et al., 1980) or any other sequences functioning in a like manner.

Man-made genes produced using this invention can also serve as a substrate for recombination with another nucleic acid. Likewise, a man-made gene pathway produced using this invention can also serve as a substrate for recombination with another nucleic acid. In a preferred instance, the recombination is facilitated by, or occurs at, areas of homology between the man-made intron-containing gene and a nucleic acid which serves as a recombination partner. In a particularly preferred instance, the recombination partner may also be a nucleic acid generated by this invention, including a man-made gene or a man-made gene pathway. Recombination may be facilitated by or may occur at areas of homology that exist at the one (or more) artificially introduced intron(s) in the man-made gene.

The synthetic ligation reassembly method of this invention utilizes a plurality of nucleic acid building blocks, each of which preferably has two ligatable ends. The two ligatable ends on each nucleic acid building block may be two blunt ends (i.e. each having an overhang of zero nucleotides), or preferably one blunt end and one overhang, or more preferably still two overhangs.

A serviceable overhang for this purpose may be a 3' overhang or a 5' overhang. Thus, a nucleic acid building block may have a 3' overhang or alternatively a 5' overhang or alternatively two 3' overhangs or alternatively two 5' overhangs. The overall order in which the nucleic acid building blocks are assembled to form a finalized chimeric nucleic acid molecule is determined by purposeful experimental design and is not random.

According to one preferred embodiment, a nucleic acid building block is generated by chemical synthesis of two single-stranded nucleic acids (also referred to as single-stranded oligos) and contacting them so as to allow them to anneal to form a double-stranded nucleic acid building block.

A double-stranded nucleic acid building block can be of variable size. The sizes of these building blocks can be small or large depending on the choice of the experimenter. Preferred sizes for building block range from 1 base pair (not including any overhangs) to 100,000 base pairs (not including any overhangs). Other preferred size ranges are also provided, which have lower limits of from 1 bp to 10,000 bp (including every integer value in between), and upper limits of from 2 bp to 100, 000 bp (including every integer value in between).

It is appreciated that current methods of polymerase-based amplification can be used to generate double-stranded nucleic acids of up to thousands of base pairs, if not tens of thousands of base pairs, in length with high fidelity. Chemical synthesis (e.g. phosphoramidite-based) can be used to generate nucleic acids of up to hundreds of nucleotides in length with high fidelity; however, these can be assembled, e.g. using overhangs or sticky ends, to form double-stranded nucleic acids of up to thousands of base pairs, if not tens of thousands of base pairs, in length if so desired.

A combination of methods (e.g. phosphoramidite-based chemical synthesis and PCR) can also be used according to this invention. Thus, nucleic acid building block made by different methods can also be used in combination to generate a progeny molecule of this invention.

The use of chemical synthesis to generate nucleic acid building blocks is particularly preferred in this invention & is advantageous for other reasons as well, including procedural safety and ease. No cloning or harvesting or actual handling of any biological samples is required. The design of the nucleic acid building blocks can be accomplished on paper. Accordingly, this invention teaches an advance in procedural safety in recombinant technologies.

Nonetheless, according to one preferred embodiment, a double-stranded nucleic acid building block according to this invention may also be generated by polymerase-based amplification of a polynucleotide template. In a non-limiting exemplification, as illustrated in Figure 2, a first polymerase-based amplification reaction using a first set of

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- 1) The design of specific nucleic acid building blocks.
 - 2) The design of specific ligatable ends on each nucleic acid building block.
 - 3) The design of a particular order of assembly of the nucleic acid building blocks.

An overhang may be a 3' overhang or a 5' overhang. An overhang may also have a terminal phosphate group or alternatively may be devoid of a terminal phosphate group (having, e.g., a hydroxyl group instead). An overhang may be comprised of any number of nucleotides. Preferably an overhang is comprised of 0 nucleotides (as in a blunt end) to 10,000 nucleotides. Thus, a wide range of overhang sizes may be serviceable. Accordingly, the lower limit may be each integer from 1-200 and the upper limit may be each integer from 2-10,000. According to a particular exemplification, an overhang may consist of anywhere from 1 nucleotide to 200 nucleotides (including every integer value in between).

The final chimeric nucleic acid molecule may be generated by sequentially assembling 2 or more building blocks at a time until all the designated building blocks have been assembled. A working sample may optionally be subjected to a process for size selection or purification or other selection or enrichment process between the performance of two assembly steps. Alternatively, the final chimeric nucleic acid molecule may be generated by assembling all the designated building blocks at once in one step.

Utility

The *in vivo* recombination method of this invention can be performed blindly on a pool of unknown hybrids or alleles of a specific polynucleotide or sequence. However, it is not necessary to know the actual DNA or RNA sequence of the specific polynucleotide.

The approach of using recombination within a mixed population of genes can be useful for the generation of any useful proteins, for example, interleukin I, antibodies, tPA and growth hormone. This approach may be used to generate proteins having altered

specificity or activity. The approach may also be useful for the generation of hybrid nucleic acid sequences, for example, promoter regions, introns, exons, enhancer sequences, 3' untranslated regions or 5' untranslated regions of genes. Thus this approach may be used to generate genes having increased rates of expression. This approach may also be useful in the study of repetitive DNA sequences. Finally, this approach may be useful to mutate ribozymes or aptamers.

Scaffold-like regions separating regions of diversity in proteins may be particularly suitable for the methods of this invention. The conserved scaffold determines the overall folding by self-association, while displaying relatively unrestricted loops that mediate the specific binding. Examples of such scaffolds are the immunoglobulin beta barrel, and the four-helix bundle. The methods of this invention can be used to create scaffold-like proteins with various combinations of mutated sequences for binding.

The equivalents of some standard genetic matings may also be performed by the methods of this invention. For example, a "molecular" backcross can be performed by repeated mixing of the hybrid's nucleic acid with the wild-type nucleic acid while selecting for the mutations of interest. As in traditional breeding, this approach can be used to combine phenotypes from different sources into a background of choice. It is useful, for example, for the removal of neutral mutations that affect unselected characteristics (i.e. immunogenicity). Thus it can be useful to determine which mutations in a protein are involved in the enhanced biological activity and which are not.

3.2.4. END-SELECTION

This invention provides a method for selecting a subset of polynucleotides from a starting set of polynucleotides, which method is based on the ability to discriminate one or more selectable features (or selection markers) present anywhere in a working polynucleotide, so as to allow one to perform selection for (positive selection) &/or against (negative selection) each selectable polynucleotide. In a preferred aspect, a method is provided termed end-selection, which method is based on the use of a selection marker located in part or entirely in a terminal region of a selectable polynucleotide, and such a selection marker may be termed an "end-selection marker".

End-selection may be based on detection of naturally occurring sequences or on detection of sequences introduced experimentally (including by any mutagenesis procedure mentioned herein and not mentioned herein) or on both, even within the same polynucleotide. An end-selection marker can be a structural selection marker or a functional selection marker or both a structural and a functional selection marker. An end-selection marker may be comprised of a polynucleotide sequence or of a polypeptide sequence or of any chemical structure or of any biological or biochemical tag, including markers that can be selected using methods based on the detection of radioactivity, of enzymatic activity, of fluorescence, of any optical feature, of a magnetic property (e.g. using magnetic beads), of immunoreactivity, and of hybridization.

End-selection may be applied in combination with any method serviceable for performing mutagenesis. Such mutagenesis methods include, but are not limited to, methods described herein (supra and infra). Such methods include, by way of non-limiting exemplification, any method that may be referred herein or by others in the art by any of the following terms: "saturation mutagenesis", "shuffling", "recombination", "re-assembly", "error-prone PCR", "assembly PCR", "sexual PCR", "crossover PCR", "oligonucleotide primer-directed mutagenesis", "recursive (&/or exponential) ensemble mutagenesis (see **Arkin and Youvan, 1992**)", "cassette mutagenesis", "in vivo mutagenesis", and "in vitro mutagenesis". Moreover, end-selection may be performed on molecules produced by any mutagenesis &/or amplification method (see, e.g., **Arnold, 1993; Caldwell and Joyce, 1992; Stemmer, 1994**; following which method it is desirable to select for (including to screen for the presence of) desirable progeny molecules.

In addition, end-selection may be applied to a polynucleotide apart from any mutagenesis method. In a preferred embodiment, end-selection, as provided herein, can be used in order to facilitate a cloning step, such as a step of ligation to another polynucleotide (including ligation to a vector). This invention thus provides for end-selection as a serviceable means to facilitate library construction, selection &/or enrichment for desirable polynucleotides, and cloning in general.

In a particularly preferred embodiment, end-selection can be based on (positive) selection for a polynucleotide; alternatively end-selection can be based on (negative) selection against a polynucleotide; and alternatively still, end-selection can be based on both (positive) selection for, and on (negative) selection against, a polynucleotide. End-selection, along with other methods of selection &/or screening, can be performed in an iterative fashion, with any combination of like or unlike selection &/or screening methods and serviceable mutagenesis methods, all of which can be performed in an iterative fashion and in any order, combination, and permutation.

It is also appreciated that, according to one embodiment of this invention, end-selection may also be used to select a polynucleotide that is at least in part: circular (e.g. a plasmid or any other circular vector or any other polynucleotide that is partly circular), &/or branched, &/or modified or substituted with any chemical group or moiety. In accord with this embodiment, a polynucleotide may be a circular molecule comprised of an intermediate or central region, which region is flanked on a 5' side by a 5' flanking region (which, for the purpose of end-selection, serves in like manner to a 5' terminal region of a non-circular polynucleotide) and on a 3' side by a 3' terminal region (which, for the purpose of end-selection, serves in like manner to a 3' terminal region of a non-circular polynucleotide). As used in this non-limiting exemplification, there may be sequence overlap between any two regions or even among all three regions.

In one non-limiting aspect of this invention, end-selection of a linear polynucleotide is performed using a general approach based on the presence of at least one end-selection marker located at or near a polynucleotide end or terminus (that can be either a 5' end or a 3' end). In one particular non-limiting exemplification, end-selection is based on selection for a specific sequence at or near a terminus such as, but not limited to, a sequence recognized by an enzyme that recognizes a polynucleotide sequence. An enzyme that recognizes and catalyzes a chemical modification of a polynucleotide is referred to herein as a polynucleotide-acting enzyme. In a preferred embodiment, serviceable polynucleotide-acting enzymes are exemplified non-exclusively by enzymes with polynucleotide-cleaving activity, enzymes with polynucleotide-methylating activity, enzymes with polynucleotide-ligating activity, and enzymes with a plurality of

distinguishable enzymatic activities (including non-exclusively, e.g., both polynucleotide-cleaving activity and polynucleotide-ligating activity).

Relevant polynucleotide-acting enzymes thus also include any commercially available or non-commercially available polynucleotide endonucleases and their companion methylases including those catalogued at the website <http://www.neb.com/rebase>, and those mentioned in the following cited reference (Roberts and Macelis, 1996). Preferred polynucleotide endonucleases include – but are not limited to – type II restriction enzymes (including type IIS), and include enzymes that cleave both strands of a double stranded polynucleotide (e.g. *Not* I, which cleaves both strands at 5'...GC/GGCCGC...3') and enzymes that cleave only one strand of a double stranded polynucleotide, i.e. enzymes that have polynucleotide-nicking activity, (e.g. *N. Bst*NB I, which cleaves only one strand at 5'...GAGTCNNNN/N...3'). Relevant polynucleotide-acting enzymes also include type III restriction enzymes.

It is appreciated that relevant polynucleotide-acting enzymes also include any enzymes that may be developed in the future, though currently unavailable, that are serviceable for generating a ligation compatible end, preferably a sticky end, in a polynucleotide.

In one preferred exemplification, a serviceable selection marker is a restriction site in a polynucleotide that allows a corresponding type II (or type IIS) restriction enzyme to cleave an end of the polynucleotide so as to provide a ligatable end (including a blunt end or alternatively a sticky end with at least a one base overhang) that is serviceable for a desirable ligation reaction without cleaving the polynucleotide internally in a manner that destroys a desired internal sequence in the polynucleotide. Thus it is provided that, among relevant restriction sites, those sites that do not occur internally (i.e. that do not occur apart from the termini) in a specific working polynucleotide are preferred when the use of a corresponding restriction enzyme(s) is not intended to cut the working polynucleotide internally. This allows one to perform restriction digestion reactions to completion or to near completion without incurring unwanted internal cleavage in a working polynucleotide.

According to a preferred aspect, it is thus preferable to use restriction sites that are not contained, or alternatively that are not expected to be contained, or alternatively that are unlikely to be contained (e.g. when sequence information regarding a working polynucleotide is incomplete) internally in a polynucleotide to be subjected to end-selection. In accordance with this aspect, it is appreciated that restriction sites that occur relatively infrequently are usually preferred over those that occur more frequently. On the other hand it is also appreciated that there are occasions where internal cleavage of a polypeptide is desired, e.g. to achieve recombination or other mutagenic procedures along with end-selection.

In accord with this invention, it is also appreciated that methods (e.g. mutagenesis methods) can be used to remove unwanted internal restriction sites. It is also appreciated that a partial digestion reaction (i.e. a digestion reaction that proceeds to partial completion) can be used to achieve digestion at a recognition site in a terminal region while sparing a susceptible restriction site that occurs internally in a polynucleotide and that is recognized by the same enzyme. In one aspect, partial digest are useful because it is appreciated that certain enzymes show preferential cleavage of the same recognition sequence depending on the location and environment in which the recognition sequence occurs. For example, it is appreciated that, while lambda DNA has 5 *EcoR* I sites, cleavage of the site nearest to the right terminus has been reported to occur 10 times faster than the sites in the middle of the molecule. Also, for example, it has been reported that, while *Sac* II has four sites on lambda DNA, the three clustered centrally in lambda are cleaved 50 times faster than the remaining site near the terminus (at nucleotide 40,386). Summarily, site preferences have been reported for various enzymes by many investigators (e.g., **Thomas and Davis**, 1975; **Forsblum et al**, 1976; **Nath and Azzolina**, 1981; **Brown and Smith**, 1977; **Gingeras and Brooks**, 1983; **Krüger et al**, 1988; **Conrad and Topal**, 1989; **Oller et al**, 1991; **Topal**, 1991; and **Pein**, 1991; to name but a few). It is appreciated that any empirical observations as well as any mechanistic understandings of site preferences by any serviceable polynucleotide-acting enzymes, whether currently available or to be procured in the future, may be serviceable in end-selection according to this invention.

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It is also appreciated that protection methods can be used to selectively protect specified restriction sites (e.g. internal sites) against unwanted digestion by enzymes that would otherwise cut a working polypeptide in response to the presence of those sites; and that such protection methods include modifications such as methylations and base substitutions (e.g. U instead of T) that inhibit an unwanted enzyme activity. It is appreciated that there are limited numbers of available restriction enzymes that are rare enough (e.g. having very long recognition sequences) to create large (e.g. megabase-long) restriction fragments, and that protection approaches (e.g. by methylation) are serviceable for increasing the rarity of enzyme cleavage sites. The use of *M.Fnu* II (mCGCG) to increase the apparent rarity of *Not* I approximately twofold is but one example among many (Qiang et al, 1990; Nelson et al, 1984; Maxam and Gilbert, 1980; Raleigh and Wilson, 1986).

According to a preferred aspect of this invention, it is provided that, in general, the use of rare restriction sites is preferred. It is appreciated that, in general, the frequency of occurrence of a restriction site is determined by the number of nucleotides contained therein, as well as by the ambiguity of the base requirements contained therein. Thus, in a non-limiting exemplification, it is appreciated that, in general, a restriction site composed of, for example, 8 specific nucleotides (e.g. the *Not* I site or GC/GGCCGC, with an estimated relative occurrence of 1 in 4^8 , i.e. 1 in 65,536, random 8-mers) is relatively more infrequent than one composed of, for example, 6 nucleotides (e.g. the *Sma* I site or CCC/GGG, having an estimated relative occurrence of 1 in 4^6 , i.e. 1 in 4,096, random 6-mers), which in turn is relatively more infrequent than one composed of, for example, 4 nucleotides (e.g. the *Msp* I site or C/CGG, having an estimated relative occurrence of 1 in 4^4 , i.e. 1 in 256, random 4-mers). Moreover, in another non-limiting exemplification, it is appreciated that, in general, a restriction site having no ambiguous (but only specific) base requirements (e.g. the *Fin* I site or GTCCC, having an estimated relative occurrence of 1 in 4^5 , i.e. 1 in 1024, random 5-mers) is relatively more infrequent than one having an ambiguous W (where W = A or T) base requirement (e.g. the *Ava* II site or G/GWCC, having an estimated relative occurrence of 1 in $4 \times 4 \times 2 \times 4 \times 4$ - i.e. 1 in 512 - random 5-mers), which in turn is relatively more infrequent than one having an ambiguous N (where N = A or C or G or T) base requirement (e.g. the *Asu* I site or G/GNCC, having an estimated relative occurrence of 1 in $4 \times 4 \times 1 \times 4 \times 4$, i.e. 1 in 256 - random 5-mers). These

relative occurrences are considered general estimates for actual polynucleotides, because it is appreciated that specific nucleotide bases (not to mention specific nucleotide sequences) occur with dissimilar frequencies in specific polynucleotides, in specific species of organisms, and in specific groupings of organisms. For example, it is appreciated that the % G+C contents of different species of organisms are often very different and wide ranging.

The use of relatively more infrequent restriction sites as a selection marker include - in a non-limiting fashion - preferably those sites composed at least a 4 nucleotide sequence, more preferably those composed of at least a 5 nucleotide sequence, more preferably still those composed at least a 6 nucleotide sequence (e.g. the *Bam*H I site or G/GATCC, the *Bgl* II site or A/GATCT, the *Pst* I site or CTGCA/G, and the *Xba* I site or T/CTAGA), more preferably still those composed at least a 7 nucleotide sequence, more preferably still those composed of an 8 nucleotide sequence (e.g. the *Asc* I site or GG/CGCGCC, the *Not* I site or GC/GGCCGC, the *Pac* I site or TTAAT/TAA, the *Pme* I site or GTTT/AAAC, the *Srf* I site or GCCC/GGGC, the *Sse*838 I site or CCTGCA/GG, and the *Swa* I site or ATTT/AAAT), more preferably still those composed of a 9 nucleotide sequence, and even more preferably still those composed of at least a 10 nucleotide sequence (e.g. the *Bsp*G I site or CG/CGCTGGAC). It is further appreciated that some restriction sites (e.g. for class IIS enzymes) are comprised of a portion of relatively high specificity (i.e. a portion containing a principal determinant of the frequency of occurrence of the restriction site) and a portion of relatively low specificity; and that a site of cleavage may or may not be contained within a portion of relatively low specificity. For example, in the *Eco*57 I site or CTGAAG(16/14), there is a portion of relatively high specificity (i.e. the CTGAAG portion) and a portion of relatively low specificity (i.e. the N16 sequence) that contains a site of cleavage.

In another preferred embodiment of this invention, a serviceable end-selection marker is a terminal sequence that is recognized by a polynucleotide-acting enzyme that recognizes a specific polynucleotide sequence. In a preferred aspect of this invention, serviceable polynucleotide-acting enzymes also include other enzymes in addition to classic type II restriction enzymes. According to this preferred aspect of this invention,

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In a particularly preferred exemplification that is non-limiting yet clearly illustrative, it is appreciated that when a nicking site for topoisomerase-based end-selection is 4 nucleotides from a terminus, nicking produces a single stranded oligo of 4 bases (in a terminal region) that can be denatured from its complementary strand in an end-selectable polynucleotide; this provides a sticky end (comprised of 4 bases) in a polynucleotide that is serviceable for an ensuing ligation reaction. To accomplish ligation to a cloning vector (preferably an expression vector), compatible sticky ends can be

generated in a cloning vector by any means including by restriction enzyme-based means. The terminal nucleotides (comprised of 4 terminal bases in this specific example) in an end-selectable polynucleotide terminus are thus wisely chosen to provide compatibility with a sticky end generated in a cloning vector to which the polynucleotide is to be ligated.

On the other hand, internal nicking of an end-selectable polynucleotide, e.g. 500 bases from a terminus, produces a single stranded oligo of 500 bases that is not easily denatured from its complementary strand, but rather is serviceable for repair (e.g. by the same topoisomerase enzyme that produced the nick).

This invention thus provides a method - e.g. that is vaccinia topoisomerase-based &/or type II (or IIS) restriction endonuclease-based &/or type III restriction endonuclease-based &/or nicking enzyme-based (e.g. using N. *Bst*NB I) - for producing a sticky end in a working polynucleotide, which end is ligation compatible, and which end can be comprised of at least a 1 base overhang. Preferably such a sticky end is comprised of at least a 2-base overhang, more preferably such a sticky end is comprised of at least a 3-base overhang, more preferably still such a sticky end is comprised of at least a 4-base overhang, even more preferably still such a sticky end is comprised of at least a 5-base overhang, even more preferably still such a sticky end is comprised of at least a 6-base overhang. Such a sticky end may also be comprised of at least a 7-base overhang, or at least an 8-base overhang, or at least a 9-base overhang, or at least a 10-base overhang, or at least 15-base overhang, or at least a 20-base overhang, or at least a 25-base overhang, or at least a 30-base overhang. These overhangs can be comprised of any bases, including A, C, G, or T.

It is appreciated that sticky end overhangs introduced using topoisomerase or a nicking enzyme (e.g. using N. *Bst*NB I) can be designed to be unique in a ligation environment, so as to prevent unwanted fragment reassemblies, such as self-dimerizations and other unwanted concatamerizations.

According to one aspect of this invention, a plurality of sequences (which may but do not necessarily overlap) can be introduced into a terminal region of an end-selectable polynucleotide by the use of an oligo in a polymerase-based reaction. In a relevant, but by

no means limiting example, such an oligo can be used to provide a preferred 5' terminal region that is serviceable for topoisomerase I-based end-selection, which oligo is comprised of: a 1-10 base sequence that is convertible into a sticky end (preferably by a vaccinia topoisomerase I), a ribosome binding site (i.e. and "RBS", that is preferably serviceable for expression cloning), and optional linker sequence followed by an ATG start site and a template-specific sequence of 0-100 bases (to facilitate annealment to the template in the polymerase-based reaction). Thus, according to this example, a serviceable oligo (which may be termed a forward primer) can have the sequence: 5'[terminal sequence = (N)₁₋₁₀][topoisomerase I site & RBS = AAGGGAGGAG][linker = (N)₁₋₁₀₀][start codon and template-specific sequence = ATG(N)₀₋₁₀₀]3'.

Analogously, in a relevant, but by no means limiting example, an oligo can be used to provide a preferred 3' terminal region that is serviceable for topoisomerase I-based end-selection, which oligo is comprised of: a 1-10 base sequence that is convertible into a sticky end (preferably by a vaccinia topoisomerase I), and optional linker sequence followed by a template-specific sequence of 0-100 bases (to facilitate annealment to the template in the polymerase-based reaction). Thus, according to this example, a serviceable oligo (which may be termed a reverse primer) can have the sequence: 5'[terminal sequence = (N)₁₋₁₀][topoisomerase I site = AAGGG][linker = (N)₁₋₁₀₀][template-specific sequence = (N)₀₋₁₀₀]3'.

It is appreciated that, end-selection can be used to distinguish and separate parental template molecules (e.g. to be subjected to mutagenesis) from progeny molecules (e.g. generated by mutagenesis). For example, a first set of primers, lacking in a topoisomerase I recognition site, can be used to modify the terminal regions of the parental molecules (e.g. in polymerase-based amplification). A different second set of primers (e.g. having a topoisomerase I recognition site) can then be used to generate mutated progeny molecules (e.g. using any polynucleotide chimerization method, such as interrupted synthesis, template-switching polymerase-based amplification, or interrupted synthesis; or using saturation mutagenesis; or using any other method for introducing a topoisomerase I recognition site into a mutagenized progeny molecule as disclosed herein) from the amplified template molecules. The use of topoisomerase I-based end-selection can then facilitate, not only discernment, but selective topoisomerase I-based ligation of the desired progeny molecules.

Annealment of a second set of primers to thusly amplified parental molecules can be facilitated by including sequences in a first set of primers (i.e. primers used for amplifying a set parental molecules) that are similar to a topoisomerase I recognition site, yet different enough to prevent functional topoisomerase I enzyme recognition. For example, sequences that diverge from the AAGGG site by anywhere from 1 base to all 5 bases can be incorporated into a first set of primers (to be used for amplifying the parental templates prior to subjection to mutagenesis). In a specific, but non-limiting aspect, it is thus provided that a parental molecule can be amplified using the following exemplary – but by no means limiting – set of forward and reverse primers:

Forward Primer: 5' CTAGAAGAGAGGAGAAAACCATG(N)₁₀₋₁₀₀ 3', and

Reverse Primer: 5' GATCAAAGGCGCGCCTGCAGG(N)₁₀₋₁₀₀ 3'

According to this specific example of a first set of primers, (N)₁₀₋₁₀₀ represents preferably a 10 to 100 nucleotide-long template-specific sequence, more preferably a 10 to 50 nucleotide-long template-specific sequence, more preferably still a 10 to 30 nucleotide-long template-specific sequence, and even more preferably still a 15 to 25 nucleotide-long template-specific sequence.

According to a specific, but non-limiting aspect, it is thus provided that, after this amplification (using a disclosed first set of primers lacking in a true topoisomerase I recognition site), amplified parental molecules can then be subjected to mutagenesis using one or more sets of forward and reverse primers that do have a true topoisomerase I recognition site. In a specific, but non-limiting aspect, it is thus provided that a parental molecule can be used as templates for the generation of a mutagenized progeny molecule using the following exemplary – but by no means limiting – second set of forward and reverse primers:

Forward Primer: 5' CTAGAAGGGAGGAGAAAACCATG 3'

Reverse Primer: 5' GATCAAAGGCGCGCCTGCAGG 3' (contains *Asc* I recognition sequence)

It is appreciated that any number of different primers sets not specifically mentioned can be used as first, second, or subsequent sets of primers for end-selection consistent with this invention. Notice that type II restriction enzyme sites can be incorporated (e.g. an *Asc* I site in the above example). It is provided that, in addition to the other sequences mentioned, the experimentalist can incorporate one or more N,N,G/T triplets into a serviceable primer in order to subject a working polynucleotide to saturation mutagenesis. Summarily, use of a second and/or subsequent set of primers can achieve dual goals of introducing a topoisomerase I site and of generating mutations in a progeny polynucleotide.

Thus, according to one use provided, a serviceable end-selection marker is an enzyme recognition site that allows an enzyme to cleave (including nick) a polynucleotide at a specified site, to produce a ligation-compatible end upon denaturation of a generated single stranded oligo. Ligation of the produced polynucleotide end can then be accomplished by the same enzyme (e.g. in the case of vaccinia virus topoisomerase I), or alternatively with the use of a different enzyme. According to one aspect of this invention, any serviceable end-selection markers, whether like (e.g. two vaccinia virus topoisomerase I recognition sites) or unlike (e.g. a class II restriction enzyme recognition site and a vaccinia virus topoisomerase I recognition site) can be used in combination to select a polynucleotide. Each selectable polynucleotide can thus have one or more end-selection markers, and they can be like or unlike end-selection markers. In a particular aspect, a plurality of end-selection markers can be located on one end of a polynucleotide and can have overlapping sequences with each other.

It is important to emphasize that any number of enzymes, whether currently in existence or to be developed, can be serviceable in end-selection according to this invention. For example, in a particular aspect of this invention, a nicking enzyme (e.g. N. *Bst*NB I, which cleaves only one strand at 5'...GAGTCNNNN/N...3') can be used in conjunction with a source of polynucleotide-ligating activity in order to achieve end-selection. According to this embodiment, a recognition site for N. *Bst*NB I – instead of a recognition site for topoisomerase I – should be incorporated into an end-selectable polynucleotide (whether end-selection is used for selection of a mutagenized progeny molecule or whether end-selection is used apart from any mutagenesis procedure).

It is appreciated that the instantly disclosed end-selection approach using topoisomerase-based nicking and ligation has several advantages over previously available selection methods. In sum, this approach allows one to achieve direction cloning (including expression cloning). Specifically, this approach can be used for the achievement of: direct ligation (i.e. without subjection to a classic restriction-purification-ligation reaction, that is susceptible to a multitude of potential problems from an initial restriction reaction to a ligation reaction dependent on the use of T4 DNA ligase); separation of progeny molecules from original template molecules (e.g. original template molecules lack topoisomerase I sites that not introduced until after mutagenesis), obviation of the need for size separation steps (e.g. by gel chromatography or by other electrophoretic means or by the use of size-exclusion membranes), preservation of internal sequences (even when topoisomerase I sites are present), obviation of concerns about unsuccessful ligation reactions (e.g. dependent on the use of T4 DNA ligase, particularly in the presence of unwanted residual restriction enzyme activity), and facilitated expression cloning (including obviation of frame shift concerns). Concerns about unwanted restriction enzyme-based cleavages – especially at internal restriction sites (or even at often unpredictable sites of unwanted star activity) in a working polynucleotide – that are potential sites of destruction of a working polynucleotide can also be obviated by the instantly disclosed end-selection approach using topoisomerase-based nicking and ligation.

3.3. ADDITIONAL SCREENING METHODS

Peptide Display Methods

The present method can be used to shuffle, by *in vitro* and/or *in vivo* recombination by any of the disclosed methods, and in any combination, polynucleotide sequences selected by peptide display methods, wherein an associated polynucleotide encodes a displayed peptide which is screened for a phenotype (e.g., for affinity for a predetermined receptor (ligand)).

An increasingly important aspect of bio-pharmaceutical drug development and molecular biology is the identification of peptide structures, including the primary amino acid sequences, of peptides or peptidomimetics that interact with biological

macromolecules. one method of identifying peptides that possess a desired structure or functional property, such as binding to a predetermined biological macromolecule (e.g., a receptor), involves the screening of a large library or peptides for individual library members which possess the desired structure or functional property conferred by the amino acid sequence of the peptide.

In addition to direct chemical synthesis methods for generating peptide libraries, several recombinant DNA methods also have been reported. One type involves the display of a peptide sequence, antibody, or other protein on the surface of a bacteriophage particle or cell. Generally, in these methods each bacteriophage particle or cell serves as an individual library member displaying a single species of displayed peptide in addition to the natural bacteriophage or cell protein sequences. Each bacteriophage or cell contains the nucleotide sequence information encoding the particular displayed peptide sequence; thus, the displayed peptide sequence can be ascertained by nucleotide sequence determination of an isolated library member.

A well-known peptide display method involves the presentation of a peptide sequence on the surface of a filamentous bacteriophage, typically as a fusion with a bacteriophage coat protein. The bacteriophage library can be incubated with an immobilized, predetermined macromolecule or small molecule (e.g., a receptor) so that bacteriophage particles which present a peptide sequence that binds to the immobilized macromolecule can be differentially partitioned from those that do not present peptide sequences that bind to the predetermined macromolecule. The bacteriophage particles (i.e., library members) which are bound to the immobilized macromolecule are then recovered and replicated to amplify the selected bacteriophage sub-population for a subsequent round of affinity enrichment and phage replication. After several rounds of affinity enrichment and phage replication, the bacteriophage library members that are thus selected are isolated and the nucleotide sequence encoding the displayed peptide sequence is determined, thereby identifying the sequence(s) of peptides that bind to the predetermined macromolecule (e.g., receptor). Such methods are further described in PCT patent publications **WO 91/17271**, **WO 91/18980**, **WO 91/19818** and **WO 93/08278**.

[illegible][illegible]

Introduction

[illegible]

Typically, these "affinity sharpening" mutations cluster in specific areas of the variable region, most commonly in the complementarity-determining regions (CDRs).

In order to overcome many of the limitations in producing and identifying high-affinity immunoglobulins through antigen-stimulated β cell development (i.e., immunization), various prokaryotic expression systems have been developed that can be manipulated to produce combinatorial antibody libraries which may be screened for high-affinity antibodies to specific antigens. Recent advances in the expression of antibodies in *Escherichia coli* and bacteriophage systems (see "alternative peptide display methods", *infra*) have raised the possibility that virtually any specificity can be obtained by either cloning antibody genes from characterized hybridomas or by de novo selection using antibody gene libraries (e.g., from Ig cDNA).

Combinatorial libraries of antibodies have been generated in bacteriophage lambda expression systems which may be screened as bacteriophage plaques or as colonies of lysogens (Huse et al, 1989); Caton and Koprowski, 1990; Mullinax et al, 1990; Persson et al, 1991). Various embodiments of bacteriophage antibody display libraries and lambda phage expression libraries have been described (Kang et al, 1991; Clackson et al, 1991; McCafferty et al, 1990; Burton et al, 1991; Hoogenboom et al, 1991; Chang et al, 1991; Breitling et al, 1991; Marks et al, 1991, p. 581; Barbas et al, 1992; Hawkins and Winter, 1992; Marks et al, 1992, p. 779; Marks et al, 1992, p. 16007; and Lowman et al, 1991; Lerner et al, 1992; all incorporated herein by reference). Typically, a bacteriophage antibody display library is screened with a receptor (e.g., polypeptide, carbohydrate, glycoprotein, nucleic acid) that is immobilized (e.g., by covalent linkage to a chromatography resin to enrich for reactive phage by affinity chromatography) and/or labeled (e.g., to screen plaque or colony lifts).

One particularly advantageous approach has been the use of so-called single-chain fragment variable (scfv) libraries (Marks et al, 1992, p. 779; Winter and Milstein, 1991; Clackson et al, 1991; Marks et al, 1991, p. 581; Chaudhary et al, 1990; Chiswell et al, 1992; McCafferty et al, 1990; and Huston et al, 1988). Various embodiments of scfv libraries displayed on bacteriophage coat proteins have been described.

Despite these substantial limitations, bacteriophage display of scfv have already yielded a variety of useful antibodies and antibody fusion proteins. A bispecific single chain antibody has been shown to mediate efficient tumor cell lysis (**Gruber et al, 1994**). Intracellular expression of an anti-Rev scfv has been shown to inhibit HIV-1 virus replication *in vitro* (**Duan et al, 1994**), and intracellular expression of an anti-p21^{ras}, scfv has been shown to inhibit meiotic maturation of Xenopus oocytes (**Biocca et al, 1993**). Recombinant scfv which can be used to diagnose HIV infection have also been reported, demonstrating the diagnostic utility of scfv (**Lilley et al, 1994**). Fusion proteins wherein an scFv is linked to a second polypeptide, such as a toxin or fibrinolytic activator protein, have also been reported (**Holvost et al, 1992; Nicholls et al, 1993**).

If it were possible to generate scfv libraries having broader antibody diversity and overcoming many of the limitations of conventional CDR mutagenesis and randomization methods which can cover only a very tiny fraction of the potential sequence combinations, the number and quality of scfv antibodies suitable for therapeutic and diagnostic use could be vastly improved. To address this, the *in vitro* and *in vivo* shuffling methods of the invention are used to recombine CDRs which have been obtained (typically via PCR amplification or cloning) from nucleic acids obtained from selected displayed antibodies. Such displayed antibodies can be displayed on cells, on bacteriophage particles, on polysomes, or any suitable antibody display system wherein the antibody is associated with its encoding nucleic acid(s). In a variation, the CDRs are initially obtained from mRNA (or cDNA) from antibody-producing cells (e.g., plasma cells/splenocytes from an immunized wild-type mouse, a human, or a transgenic mouse capable of making a human antibody as in **WO 92/03918, WO 93/12227, and WO 94/25585**), including hybridomas derived therefrom.

Polynucleotide sequences selected in a first selection round (typically by affinity selection for displayed antibody binding to an antigen (e.g., a ligand) by any of these methods are pooled and the pool(s) is/are shuffled by *in vitro* and/or *in vivo* recombination, especially shuffling of CDRs (typically shuffling heavy chain CDRs with other heavy chain CDRs and light chain CDRs with other light chain CDRs) to produce a shuffled pool comprising a population of recombined selected polynucleotide sequences.

The recombined selected polynucleotide sequences are expressed in a selection format as a displayed antibody and subjected to at least one subsequent selection round. The polynucleotide sequences selected in the subsequent selection round(s) can be used directly, sequenced, and/or subjected to one or more additional rounds of shuffling and subsequent selection until an antibody of the desired binding affinity is obtained. Selected sequences can also be back-crossed with polynucleotide sequences encoding neutral antibody framework sequences (i.e., having insubstantial functional effect on antigen binding), such as for example by back-crossing with a human variable region framework to produce human-like sequence antibodies. Generally, during back-crossing subsequent selection is applied to retain the property of binding to the predetermined antigen.

Alternatively, or in combination with the noted variations, the valency of the target epitope may be varied to control the average binding affinity of selected scfv library members. The target epitope can be bound to a surface or substrate at varying densities, such as by including a competitor epitope, by dilution, or by other method known to those in the art. A high density (valency) of predetermined epitope can be used to enrich for scfv library members which have relatively low affinity, whereas a low density (valency) can preferentially enrich for higher affinity scfv library members.

For generating diverse variable segments, a collection of synthetic oligonucleotides encoding random, pseudorandom, or a defined sequence kernel set of peptide sequences can be inserted by ligation into a predetermined site (e.g., a CDR). Similarly, the sequence diversity of one or more CDRs of the single-chain antibody cassette(s) can be expanded by mutating the CDR(s) with site-directed mutagenesis, CDR-replacement, and the like. The resultant DNA molecules can be propagated in a host for cloning and amplification prior to shuffling, or can be used directly (i.e., may avoid loss of diversity which may occur upon propagation in a host cell) and the selected library members subsequently shuffled.

Displayed peptide/polynucleotide complexes (library members) which encode a variable segment peptide sequence of interest or a single-chain antibody of interest are selected from the library by an affinity enrichment technique. This is accomplished by means of a immobilized macromolecule or epitope specific for the peptide sequence of

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interest, such as a receptor, other macromolecule, or other epitope species. Repeating the affinity selection procedure provides an enrichment of library members encoding the desired sequences, which may then be isolated for pooling and shuffling, for sequencing, and/or for further propagation and affinity enrichment.

The library members without the desired specificity are removed by washing. The degree and stringency of washing required will be determined for each peptide sequence or single-chain antibody of interest and the immobilized predetermined macromolecule or epitope. A certain degree of control can be exerted over the binding characteristics of the nascent peptide/DNA complexes recovered by adjusting the conditions of the binding incubation and the subsequent washing. The temperature, pH, ionic strength, divalent cations concentration, and the volume and duration of the washing will select for nascent peptide/DNA complexes within particular ranges of affinity for the immobilized macromolecule. Selection based on slow dissociation rate, which is usually predictive of high affinity, is often the most practical route. This may be done either by continued incubation in the presence of a saturating amount of free predetermined macromolecule, or by increasing the volume, number, and length of the washes. In each case, the rebinding of dissociated nascent peptide/DNA or peptide/RNA complex is prevented, and with increasing time, nascent peptide/DNA or peptide/RNA complexes of higher and higher affinity are recovered.

Additional modifications of the binding and washing procedures may be applied to find peptides with special characteristics. The affinities of some peptides are dependent on ionic strength or cation concentration. This is a useful characteristic for peptides that will be used in affinity purification of various proteins when gentle conditions for removing the protein from the peptides are required.

One variation involves the use of multiple binding targets (multiple epitope species, multiple receptor species), such that a scfv library can be simultaneously screened for a multiplicity of scfv which have different binding specificities. Given that the size of a scfv library often limits the diversity of potential scfv sequences, it is typically desirable to use scfv libraries of as large a size as possible. The time and economic considerations of generating a number of very large polysome scFv-display libraries can become

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hybridomas. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen et al, 1986), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, cytomegalovirus, SV40, Adenovirus, Bovine Papilloma Virus, and the like.

Eukaryotic DNA transcription can be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting sequences of between 10 to 300 bp that increase transcription by a promoter. Enhancers can effectively increase transcription when either 5' or 3' to the transcription unit. They are also effective if located within an intron or within the coding sequence itself. Typically, viral enhancers are used, including SV40 enhancers, cytomegalovirus enhancers, polyoma enhancers, and adenovirus enhancers. Enhancer sequences from mammalian systems are also commonly used, such as the mouse immunoglobulin heavy chain enhancer.

Mammalian expression vector systems will also typically include a selectable marker gene. Examples of suitable markers include, the dihydrofolate reductase gene (DHFR), the thymidine kinase gene (TK), or prokaryotic genes conferring drug resistance. The first two marker genes prefer the use of mutant cell lines that lack the ability to grow without the addition of thymidine to the growth medium. Transformed cells can then be identified by their ability to grow on non-supplemented media. Examples of prokaryotic drug resistance genes useful as markers include genes conferring resistance to G418, mycophenolic acid and hygromycin.

The vectors containing the DNA segments of interest can be transferred into the host cell by well-known methods, depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment, lipofection, or electroporation may be used for other cellular hosts. Other methods used to transform mammalian cells include the use of Polybrene, protoplast fusion, liposomes, electroporation, and micro-injection (see, generally, Sambrook et al, 1982 and 1989).

Once expressed, the antibodies, individual mutated immunoglobulin chains, mutated antibody fragments, and other immunoglobulin polypeptides of the invention can be purified according to standard procedures of the art, including ammonium sulfate precipitation, fraction column chromatography, gel electrophoresis and the like (see, generally, Scopes, 1982). Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically or in developing and performing assay procedures, immunofluorescent stainings, and the like (see, generally, **Lefkovits** and **Pernis**, 1979 and 1981; **Lefkovits**, 1997).

The antibodies generated by the method of the present invention can be used for diagnosis and therapy. By way of illustration and not limitation, they can be used to treat cancer, autoimmune diseases, or viral infections. For treatment of cancer, the antibodies will typically bind to an antigen expressed preferentially on cancer cells, such as erbB-2, CEA, CD33, and many other antigens and binding members well known to those skilled in the art.

Two-Hybrid Based Screening Assays

Shuffling can also be used to recombinatorially diversify a pool of selected library members obtained by screening a two-hybrid screening system to identify library members which bind a predetermined polypeptide sequence. The selected library members are pooled and shuffled by *in vitro* and/or *in vivo* recombination. The shuffled pool can then be screened in a yeast two hybrid system to select library members which bind said predetermined polypeptide sequence (e. g., and SH2 domain) or which bind an alternate predetermined polypeptide sequence (e.g., an SH2 domain from another protein species).

An approach to identifying polypeptide sequences which bind to a predetermined polypeptide sequence has been to use a so-called "two-hybrid" system wherein the predetermined polypeptide sequence is present in a fusion protein (**Chien** et al, 1991). This approach identifies protein-protein interactions *in vivo* through reconstitution of a transcriptional activator (**Fields and Song**, 1989), the yeast Gal4 transcription protein. Typically, the method is based on the properties of the yeast Gal4 protein, which consists of separable domains responsible for DNA-binding and transcriptional activation.

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Polynucleotides encoding two hybrid proteins, one consisting of the yeast Gal4 DNA-binding domain fused to a polypeptide sequence of a known protein and the other consisting of the Gal4 activation domain fused to a polypeptide sequence of a second protein, are constructed and introduced into a yeast host cell. Intermolecular binding between the two fusion proteins reconstitutes the Gal4 DNA-binding domain with the Gal4 activation domain, which leads to the transcriptional activation of a reporter gene (e.g., *lacZ*, *HIS3*) which is operably linked to a Gal4 binding site. Typically, the two-hybrid method is used to identify novel polypeptide sequences which interact with a known protein (**Silver and Hunt**, 1993; **Durfee et al**, 1993; **Yang et al**, 1992; **Luban et al**, 1993; **Hardy et al**, 1992; **Bartel et al**, 1993; and **Vojtek et al**, 1993). However, variations of the two-hybrid method have been used to identify mutations of a known protein that affect its binding to a second known protein (**Li and Fields**, 1993; **Lalo et al**, 1993; **Jackson et al**, 1993; and **Madura et al**, 1993). Two-hybrid systems have also been used to identify interacting structural domains of two known proteins (**Bardwell et al**, 1993; **Chakrabarty et al**, 1992; **Staudinger et al**, 1993; and **Milne and Weaver** 1993) or domains responsible for oligomerization of a single protein (**Iwabuchi et al**, 1993; **Bogerd et al**, 1993). Variations of two-hybrid systems have been used to study the *in vivo* activity of a proteolytic enzyme (**Dasmahapatra et al**, 1992). Alternatively, an *E. coli*/BCCP interactive screening system (**Germino et al**, 1993; **Guarente**, 1993) can be used to identify interacting protein sequences (i.e., protein sequences which heterodimerize or form higher order heteromultimers). Sequences selected by a two-hybrid system can be pooled and shuffled and introduced into a two-hybrid system for one or more subsequent rounds of screening to identify polypeptide sequences which bind to the hybrid containing the predetermined binding sequence. The sequences thus identified can be compared to identify consensus sequence(s) and consensus sequence kernels.

In general, standard techniques of recombination DNA technology are described in various publications (e.g. **Sambrook et al**, 1989; **Ausubel et al**, 1987; and **Berger and Kimmel**, 1987) each of which is incorporated herein in its entirety by reference. Polynucleotide modifying enzymes were used according to the manufacturer's recommendations. Oligonucleotides were synthesized on an Applied Biosystems Inc. Model 394 DNA synthesizer using ABI chemicals. If desired, PCR amplimers for

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1. Introduction

A random priming kit which utilizes a non-proofreading polymerase (for example, Prime-It II Random Primer Labeling kit by Stratagene Cloning Systems) is utilized to generate different size polynucleotides by priming at random sites on templates which are prepared by U.V. light (as described above) and extending along the templates. The priming protocols such as described in the Prime-It II Random Primer Labeling kit may be utilized to extend the primers. The dimers formed by U.V. exposure serve as a roadblock for the extension by the non-proofreading polymerase. Thus, a pool of random size polynucleotides is present after extension with the random primers is finished.

In one embodiment, the present invention provides a method for generating libraries of displayed polypeptides or displayed antibodies suitable for affinity interaction screening or phenotypic screening. The method comprises (1) obtaining a first plurality of

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In one embodiment of the invention, the DNA adducts, or polynucleotides comprising the DNA adducts, are removed from the polynucleotides or polynucleotide pool, such as by a process including heating the solution comprising the DNA fragments prior to further processing.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following examples are to be considered illustrative and thus are not limiting of the remainder of the disclosure in any way whatsoever.

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In one aspect, the present invention relates generally to the field of transposable nucleic acid and, more particularly to production and use of a modified transposase enzyme in a system for introducing genetic changes to nucleic acid.

The present invention relates to transposable elements isolated from maize and a process for using the same to identify and isolate genes and to insert desired gene sequences into plants in a heritable manner.

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4.2. SPECIFIC METHODOLOGIES

4.2.1. Description Of Transposable Elements

Transposable genetic elements are DNA sequences, found in a wide variety of prokaryotic and eukaryotic organisms, that can move or transpose from one position to another position in a genome. In vivo, intra-chromosomal transpositions as well as transpositions between chromosomal and non-chromosomal genetic material are known. In several systems, transposition is known to be under the control of a transposase enzyme that is typically encoded by the transposable element. The genetic structures and transposition mechanisms of various transposable elements are summarized, for example, in "Transposable Genetic Elements" in "The Encyclopedia of Molecular Biology," Kendrew and Lawrence, Eds., Blackwell Science, Ltd., Oxford (1994), incorporated herein by reference.

Transposable elements (hereinafter "transposons") are natural gene transfer vectors in bacteria, yeast, *Drosophila melanogaster* and other organisms. The best documented examples of transposons in bacteria are those carrying genes that confer antibiotic resistance on the bacterium in which they reside. These transposons tend to accumulate and become a part of bacterial plasmids. The biological properties of the plasmids permit the spread of the plasmids and their passengers, e.g., drug resistance transposons, in bacterial populations.

Transposons have also been used to identify and isolate otherwise inaccessible genes (See Bingham, P. M., Kidwell, M. G. and Rubin, G. M., Cell 29:995-1004 (1982)). That is, the White locus of *Drosophila melanogaster* has been isolated by virtue of the existence of an insertion mutation at the locus caused by a transposon that has been isolated and studied using recombinant DNA technology. Such applications are receiving increasing attention in plants and animals.

The use of transposable elements as deliberate gene transfer vectors evolved from work in bacteria and yeast and, as stated above, has recently been developed into a useful research tool in *Drosophila melanogaster* (See Rubin, G. M. and Spradling, A. C., Science 218:348-353 (1982)). The basic principle on which such applications are based is that transposons are compact genetic units that contain within their sequences essentially all of

the coding information required for transposition. Although the transposition functions are only now beginning to be identified in higher organisms, in bacteria, they are known to include enzymes termed transposases, as well as molecules which regulate expression of the transposase and other genes encoding transposition-specific proteins.

Transposable elements are mobile stretches of DNA which are defined by two end terminals, usually denoted attL and attR at the left and right attachment ends respectively. Natural transposable elements contain DNA coding for transposases, and often portable genes conferring traits such as resistance to antibiotics. Some transposable elements can insert randomly into targeted DNA while others are sequence specific in their insertion sites. Transposons have been implicated as having a major role in evolution, and there is evidence for natural multifunctional enzymes having originated from the natural fusion of different protein domains.

Scientists have taken advantage of transposons to transport reporter genes for use in studying gene expression. These include transcriptional (Type I) fusions and translational (Type II) fusions. Transcriptional fusions, unlike translational fusions, place a reporter gene under the control of another promoter, but do not translationally fuse two protein domains. Translational fusions have generally been made to link a reporter gene carried inside the transposon to the translational frame of the target gene so that the reporter gene is expressed under direct control of the transcription and translation signals of the target gene of interest to study gene regulation. This requires that an open reading frame extend through the end of the transposable element to join an internal reporter protein to external translational sequences. This usually results in complete inactivation of the target gene.

4.2.2. Rational protein design

The main goal of modern protein chemistry is to be able to design proteins with desired functions. The approach taken by the majority of scientists has been called rational protein design, which is highly dependent on knowledge of protein structure in three dimensions and protein folding. While an extremely powerful tool, rational protein design is currently limited to a small subset of enzymes: those with well defined three dimensional structures. The classic examples include proteins such as subtilisin from several *Bacillus* sp. (Wells, Powers et al. Proc Natl Acad Sci USA. (1987). 84: 1219-1223.) and T4 lysozyme

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The use of protein fusion in vitro to generate hybrid (chimeric) proteins has gained importance in the development of novel or multifunctional enzyme activities. Applications include using protein domains to aid in protein purification (Sherwood. (1991). TIBTECH. 9: 1- 3.) or to tag proteins for delivery to specific cellular locations (Crozel, Lazdunski et al. (1984). FEBS Lett. 172: 183-188; Moore and Kelly. (1986). Nature. 321: 443-446; Roitsch and Lehle. (1991). Eur J Biochem. 195: 145-50.). Domain shuffling between different proteins shows promise to create protein products with unique uses, often to bind a particular enzymatic activity to a site of interest (Panayotatos, Fontaine et al. (1988). Molecular genetics of bacteria and phages: prokaryotic gene regulation. 174.), as a reporter system for protein- protein interactions (Fields and Song. (1989). Nature. 340: 245-246.), to study the relatedness of different proteins (Caramori, Albertini et al. (1991). Gene. 98: 37-44.) or as targeted pharmaceuticals (Pastan and FitzGerald. (1991). Science. 254: 1173-1177.). Finally, another promising application of protein fusion to biotechnology is in creating multi-catalytic enzymes which are important in biocatalysis since they represent an alternative to co-immobilization and chemical crosslinking to create multienzyme systems (Bulow and Mosbach. (1991). TIBTECH. 9: 226-231.).

4.2.2.2. Problems in the development of protein fusions

Unfortunately, the construction of functional hybrid proteins can require an extensive knowledge of a protein's structure and functional domains in order to select a proper site for fusion. Many attempts have failed to produce the desired properties (Bowie and Sauer. (1989). J Bio Chem. 264: 7596- 7602; Ellis, Morgan et al. (1986). Proc Natl Acad Sci, USA. 83: 8137- 8141; Guan and Rose. (1984). Cell. 37: 779-787; Hellebust, Murby et al. (1989). BioTech. 7: 165-168.). Random deletions can be made to fuse two domains, but this is typically done for only one domain at a time, and the cost and time involved in such trial and error efforts can be substantial. In addition, while some gene fusions can be used to stabilize proteins, unstable structures are often formed which are recognized by the cellular degrading machinery (Bowie and Sauer. (1989). J Bio Chem. 264: 7596-7602; Hellebust, Murby et al. (1989). BioTech. 7: 165-168.). Also, even with the advanced level of molecular biological techniques available today, cloning remains a labor-intensive procedure, the results of which are not trivially predictable.

Several tools have been developed to make the construction of protein fusions simpler. These tools include new plasmid systems with convenient restriction sites (Shapira, Chou et al. (1983). Gene. 25: 71- 82.), and a method for making gene fusions using the Polymerase Chain Reaction (PCR) so that convenient restriction sites are not required (Horton, Hunt et al. (1989). Gene. 77: 61-8.). None of these approaches, however, offers a truly simple way of making random protein fusions which eliminates the labor-intensive, trial and error aspects of traditional techniques, especially in the case when at least one of the two domains being studied has not yet been well characterized.

4.2.3. In Vitro Transposition Systems

In vitro transposition systems that utilize the particular transposable elements of bacteriophage Mu and bacterial transposon Tn10 have been described, by the research groups of Kiyoshi Mizuuchi and Nancy Kleckner, respectively.

The bacteriophage Mu system was first described by Mizuuchi, K., "In Vitro Transposition of Bacteria Phage Mu: A Biochemical Approach to a Novel Replication

Reaction," Cell:785-794 (1983) and Craigie, R. et al., "A Defined System for the DNA Strand-Transfer Reaction at the Initiation of Bacteriophage Mu Transposition: Protein and DNA Substrate Requirements," P.N.A.S. U.S.A. 82:7570-7574 (1985). The DNA donor substrate (mini-Mu) for Mu in vitro reaction normally requires six Mu transposase binding sites (three of about 30 bp at each end) and an enhancer sequence located about 1 kb from the left end. The donor plasmid must be supercoiled. Proteins required are Mu-encoded A and B proteins and host-encoded HU and IHF proteins. Lavoie, B. D, and G. Chaconas, "Transposition of phage Mu DNA," Curr. Topics Microbiol. Immunol. 204:83-99 (1995). The Mu-based system is disfavored for in vitro transposition system applications because the Mu termini are complex and sophisticated and because transposition requires additional proteins above and beyond the transposase.

The Tn10 system was described by Morisato, D. and N. Kleckner, "Tn10 Transposition and Circle Formation in vitro," Cell 51:101-111 (1987) and by Benjamin, H. W. and N. Kleckner, "Excision Of Tn10 from the Donor Site During Transposition Occurs By Flush Double-Strand Cleavages at the Transposon Termini," P.N.A.S. U.S.A. 89:4648-4652 (1992). The Tn10 system involves a supercoiled circular DNA molecule carrying the transposable element (or a linear DNA molecule plus *E. coli* IHF protein). The transposable element is defined by complex 42 bp terminal sequences with IHF binding site adjacent to the inverted repeat. In fact, even longer (81 bp) ends of Tn10 were used in reported experiments. Sakai, J. et al., "Identification and Characterization of Pre-Cleavage Synaptic Complex that is an Early Intermediate in Tn10 transposition," E.M.B.O. J. 14:4374-4383 (1995). In the Tn10 system, chemical treatment of the transposase protein is essential to support active transposition. In addition, the termini of the Tn10 element limit its utility in a generalized in vitro transposition system.

Both the Mu- and Tn10-based in vitro transposition systems are further limited in that they are active only on covalently closed circular, supercoiled DNA targets. What is desired is a more broadly applicable in vitro transposition system that utilizes shorter, more well defined termini and which is active on target DNA of any structure (linear, relaxed circular, and supercoiled circular DNA).

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Although the United States presently has an excess productivity in the agricultural sector, this is recognized as a local and short term condition. Thus, agricultural research and planning must be based on long term considerations. The variety of problems surrounding increases in population, degradation of prime farm land and decreasing availability of good farm land necessitates the increased use of marginal land, as well as exogenous fertilizers and chemical pest control compositions.

Classical plant breeding programs have thus far been successful in increasing agricultural productivity. However, a substantial fraction of the increase in farm productivity experienced in the United States in the past 40 years is attributable to the use of fertilizers and modern energy intensive cultivation practices, both of which are increasingly costly. The ability of plant breeding alone to sustain productivity is a matter of some question. Plant breeders are divided in their views on whether genetic improvements will continue at the rate that has occurred over the past few decades or will begin to level out. Since such questions cannot be resolved a priori, it is prudent to explore a variety of additional means by which agronomically useful traits can be accumulated and improved in major crop plants. The unconventional areas that are presently receiving the most attention in the academic research establishment, as well as in both small and large firms with plant-oriented research programs are wide genetic crosses, tissue culture and the development of gene transfer systems that circumvent fertility barriers.

In the past, many attempts have been made to transform plant cells with DNA from a variety of sources. The first unequivocal demonstration that DNA transfer can and does occur in plants emerged from the work described above on *Agrobacterium tumefaciens* Ti plasmid. However, Ti-plasmid mediated gene transfer is presently accomplished only in dicotyledonous plants that interact with the plasmid's natural host bacterium. Since most major crop species are monocotyledonous, ti-plasmid mediated gene transfer has limited applications.

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4.2.4.1. Use Of Transposons On The Ti Plasmid Of *Agrobacterium*

In higher organisms, transposons have been, or are being, used in several ways. For example, transposons are used as mutagens on the Ti plasmid of *Agrobacterium tumefaciens*. That is, a method for using bacterial transposons to cause insertion mutations in the *Agrobacterium tumefaciens* Ti plasmid, the causative agent of crown gall disease in dicotyledonous plants, has been developed. (See Zambriski, P., Goodman, H., Van Montagu, M. and Schell, J., *Mobile Genetic Elements*, J. Shapiro, Ed., (Academic Press) New York, pp. 506-535 (1983)). By this technique, it has become possible to identify the plasmid-borne genes that are responsible for virulence, as well as those that are responsible for the tumorous transformation of plant cells caused by the Ti plasmid. Further, it has become possible to show by using transposable elements, that a portion of the Ti plasmid can be integrated into plant genomes and can act as a vehicle for transferring genes from virtually any organism to any dicotyledonous plant that is susceptible to *Agrobacterium tumefaciens*.

4.2.4.2. Use Of Transposons In Maize

In maize, a monocotyledon, transposable elements were first genetically identified in the mid-1940s. These elements have been studied extensively and their genetic behavior has been extensively reviewed (See McClintock, B., *Cold Spring Harbor Symp. Quant. Biol.* 16:13-47 (1951); McClintock, B., *Cold Spring Harbor Symp. Quant. Biol.* 21:197-216 (1956); McClintock, B., *Brookhaven Symp. Biol.* 18:162-184 (1965); Fincham, J. R. S., and Sastry, G. R. K., *Ann. Rev. Genet.* 8:15-50 (1974); and Fedoroff, N., *Mobile Genetic Elements*, J. Shapiro, Ed., (Academic Press) New York, pp. 1-63 (1983)).

It has been demonstrated that transposons are normal, although cryptic, residents of the maize genome and that upon activation, they are responsible for various types of genetic rearrangements, including chromosome breakage, deletions, duplications, inversions and translocations. In addition, it has been shown that certain common types of unstable mutations, which have been studied for decades in both maize and in other organisms, are attributable to the insertion of transposons into genes or genetic loci.

4.2.5. Mu and the Transposing Bacteriophage

Bacteriophage Mu represents a class of transposons known as transposable bacteriophage which both function as a virus and a transposon. Mu replicates itself by transposing at high frequency, but can also integrate randomly into its host's genome as a lysogen. Mu is a model system for other transposable bacteriophage which are generally highly homologous. These include the *Pseudomonas* phage D3112, D108, and several other phage.

Because of the randomness of Mu insertions, and the high levels of transposition which can be generated by Mu strains containing a temperature sensitive transposition repressor (Mu_{ts} strains), Mu has been developed into a genetic tool to study gene expression in bacterial systems. Transposition of Mu derivatives has allowed scientists to perturb and examine the basic components critical to protein expression and translation. The most commonly used Mu derivatives include reporter genes which have been integrated into the Mu genome.

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4.2.5.1. Type I Fusions

Type I transcriptional fusions have been used to study gene expression and regulation by co-opting the native transcriptional signal to express the exogenous reporter gene. For example for gene expression in *E. coli*, yeast, and *Drosophila* development.

4.2.5.2. Type II Fusions

Type II fusions have also been used to study gene expression and regulation, but in this case not only co-opt the transcriptional signals, but any translational signals as well to express the reporter gene. In this type of system the protein product usually only expresses the activity of the reporter exogenous gene.

MudII elements are mini-Mu deletion elements which are type II Mu transposable elements. Examples of these include beta-galactosidase fusion elements, where a beta-galactosidase (*lacZ*) reporter gene is inserted via transposable elements to detect transcription and translation of regulated gene systems. This usually results in the inactivation of the targeted gene.

Two types of Mu protein fusions have been developed, *lacZ* fusion elements and *nptI* fusion elements (Symonds, Toussaint et al. (1987). Phage Mu) The *lacZ* elements have been used to study translation regulation, determination of the translation phase of target genes, infer the location of a protein fusion by hybrid protein size, determine amino terminal sequence, and raise antibodies to regions of the protein of interest. By far the major goal of these studies has been to determine mechanisms of gene expression in the studied organisms.

The *nptI* system was designed to perform transposon-tagging since *nptI* is known to function as an aminoglycoside resistance gene in a variety of organisms. Transposon tagging is a method of creating an mutant by inserting a transposon with a selectable marker into the gene of interest so that mutants which inactivate the gene can be identified and maintained. This element is useful since it allows the *nptI* to be directly linked to the transcription/translation system of the organism being studied.

In these studies there has been no emphasis on creating novel proteins with new activities using these transposable elements. More importantly, these Mu elements are restricted to making amino-terminal fusions to the reporter protein. In these cases the inserted reporter gene is fused to the carboxy-end of the truncated targeted protein, terminating inside the Mu. If the transposable element were to insert before the amino terminal of a targeted gene, functional translation could only occur on the marker gene by itself, and no translation of the target gene would occur.

4.2.5.3. Problems with Mu

Unfortunately, available Mu elements had several problems. First, it has not been demonstrated that Mu elements can be readily used as a general method for the development of fusion proteins with two active domains. Second, the Mu elements used thus far for creation of protein fusions can not be used for construction of "carboxy-terminal" fusions since they did not have an open reading frame extending into the element. Third, the Mu elements previously used have long linker regions which incorporate a 40 amino acid linker between the fused domains. This could create protein folding problems or unwanted domain interactions. Fourth the currently existing Mu elements had only a single restriction site for the insertion of protein domains. Finally, although Mu elements which had deleted ends existed, it was not known whether they would transpose well with additional sequences added in such close proximity to the right end and whether the intervening linker region which would join the two protein domains would interfere with the construction of active chimeric proteins.

4.2.6. Other transposons

Other transposons have been used in a similar manner as Mu to create lac fusions to study gene expression. These include Tn10 and Tn917 (Berg and Howe. (1989). Mobile DNA). The Tn5 element has also been used to construct phoA fusions in vivo. Fusions with alkaline phosphatase (phoA) have also been used to probe the structure of membrane bound proteins (Lloyd and Kadner. (1990). J Bacteriol. 172: 1688-93.). In general, these transposons have been used to study the membrane topology structure of a particular gene and protein secretion. The resultant fusion proteins are also limited to amino-terminal

Dedication

Groenen and van de Putte (1986), teach that the Mu A protein binds weakly to sequences between nucleotides 1 to 30 on the right end (R1) and between nucleotides 110 and 135 on the left end (L2). Mutations in these weak A binding sites have a greater effect on transposition than mutations of corresponding base pairs in the stronger A binding sites, located adjacent to these weak A binding sites. (Groenen and van de Putte. (1986). J Mol Biol. 189: 597-602.)

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4.3.1.1. Donor DNA Molecule: Transposable DNA Sequence Of Interest

4.3.1.1.1. Modified Transposase Enzyme Comprises Two Classes Of Differences From Wild Type Tn5 Transposase

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4.3.2.5. Transposable element comprising a left attachment site

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protein fusions in a target DNA. The target DNA can be plasmid DNA, DNA segments, genomic DNA, or other DNA targets. In a preferred embodiment of the instant invention, the right end of the transposable element consists of no more than 58 nucleic acid base pairs derived from the Mu family of transposable elements. The instant invention encompasses methods for using transposable element constructs with deletions in the right end for generating fusion proteins in vitro and in vivo. A method of the instant invention allows for the rapid and efficient generation of alternatively fused proteins suitable for screening for activity. In one embodiment of the method of the instant invention generates fusion proteins in which the exogenous DNA segment has been transposed such that the resultant fusion protein functionally expresses the exogenous protein as a amino- terminal fusion to the carboxy end of a targeted protein. In a further embodiment of a method of the instant invention, the transposable element causes the insertion of the exogenous DNA such that the functionally expressed fusion protein consists of the exogenous protein at the amino end, linked to the endogenous protein at the carboxy end of the fusion protein.

The instant invention provides for a method for in vivo protein fusion in a target organism genome comprising constructing a transposable element with a left attachment site attL, a polylinker, and a right attachment site of no more than 58 nucleic acid base pairs, inserting within the polylinker an exogenous DNA sequence, transposing the transposable element into a target organism genome, expanding the target organism, isolating protein, and screening for fusion protein containing the translated exogenous DNA. In a preferred embodiment, the instant invention provides for the in vivo carboxy terminal translational protein fusion in a target organism genome comprising constructing a transposable element with a left attachment site attL and a right attachment site attR of no more than 58 nucleic acid base pairs, inserting within the transposable element an exogenous DNA sequence, transposing the transposable element into a target organism genome, expanding the target organism, isolating protein, and screening for fusion protein containing the translated exogenous DNA. In another preferred embodiment, the instant invention provides for in vivo amino terminal translational protein fusion in a target organism genome comprising constructing a transposable element with a left attachment site attL and a right attachment site attR of no more than 58 nucleic acid base pairs, inserting within the transposable element an exogenous DNA sequence, transposing the transposable

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It will be appreciated that this technique provides a simple, in vitro system for introducing any transposable element from a donor DNA into a target DNA. It is generally accepted and understood that Tn5 transposition requires only a pair of OE termini, located to either side of the transposable element. These OE termini are generally thought to be 18 or 19 bases in length and are inverted repeats relative to one another. Johnson, R. C., and W. S. Reznikoff, *Nature* 304:280 (1983), incorporated herein by reference. The Tn5 inverted repeat sequences, which are referred to as "termini" even though they need not be at the termini of the donor DNA molecule, are well known and understood.

Apart from the need to flank the desired transposable element with standard Tn5 outside end ("OE") termini, few other requirements on either the donor DNA or the target DNA are envisioned. It is thought that Tn5 has few, if any, preferences for insertion sites, so it is possible to use the system to introduce desired sequences at random into target DNA. Therefore, it is believed that this method, employing the modified transposase described herein and a simple donor DNA, is broadly applicable to introduce changes into any target DNA, without regard to its nucleotide sequence. It will, thus, be applied to many problems of interest to those skilled in the art of molecular biology.

In the method, the modified transposase protein is combined in a suitable reaction buffer with the donor DNA and the target DNA. A suitable reaction buffer permits the transposition reaction to occur. A preferred, but not necessarily optimized, buffer contains spermidine to condense the DNA, glutamate, and magnesium, as well as a detergent, which is preferably 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propane sulfonate ("CHAPS"). The mixture can be incubated at a temperature greater than 0 °C and as high as about 28 °C to facilitate binding of the enzyme to the OE termini. A preferred temperature range is between 16° C and 28 ° C A most preferred pretreatment temperature is about 20 °C Under different buffer conditions, however, it may be possible to use other

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4.4.1.1.2.3.1. Inhibitor Protein

An AUG in the wild type Tn5 transposase gene that encodes methionine at transposase amino acid 56 is the first codon of the inhibitor protein. However, it has already been shown that replacement of the methionine at position 56 has no apparent effect upon the transposase activity, but at the same time prevents translation of the inhibitor protein, thus resulting in a somewhat higher transposition rate. Weigand, T. W. and W. S. Reznikoff, "Characterization of Two Hypertransposing Tn5 Mutants," J. Bact. 174:1229-1239 (1992), incorporated herein by reference. In particular, the present inventors have replaced the methionine with an alanine in the preferred embodiment (and have replaced the

methionine-encoding AUG codon with an alanine-encoding GCC). A preferred transposase of the present invention therefore includes an amino acid other than methionine at amino acid position 56, although this change can be considered merely technically advantageous (since it ensures the absence of the inhibitor from the in vitro system) and not essential to the invention (since other means can be used to eliminate the inhibitor protein from the in vitro system).

4.4.1.2. Preferred Transposase Amino Acid Sequence

The most preferred transposase amino acid sequence known to the inventors differs from the wild type at amino acid positions 54, 56, and 372. The mutations at positions 54 and 372 separately contribute approximately a 10-fold increase to the rate of transposition reaction in vivo. When the mutations are combined using standard recombinant techniques into a single molecule containing both classes of mutations, reaction rates of at least about 100-fold higher than can be achieved using wild type transposase are observed when the products of the in vitro system are tested in vivo. The mutation at position 56 does not directly affect the transposase activity.

Other mutants from wild type that are contemplated to be likely to contribute to high transposase activity in vitro include, but are not limited to glutamic acid-to-lysine at position 110, and glutamic acid to lysine at position 345.

It is, of course, understood that other changes apart from these noted positions can be made to the modified transposase (or to a construct encoding the modified transposase) without adversely affecting the transposase activity. For example, it is well understood that a construct encoding such a transposase could include changes in the third position of codons such that the encoded amino acid does not differ from that described herein. In addition, certain codon changes have little or no functional effect upon the transposition activity of the encoded protein. Finally, other changes may be introduced which provide yet higher transposition activity in the encoded protein. It is also specifically envisioned that combinations of mutations can be combined to encode a modified transposase having even higher transposition activity than has been exemplified herein. All of these changes are within the scope of the present invention. It is noted, however, that a modified transposase containing the EK110 and EK345 mutations (both described by Weigand and

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After the enzyme is prepared and purified, as described supra, it can be used in the in vitro transposition reaction described above to introduce any desired transposable element from a donor DNA into a target DNA. The donor DNA can be circular or can be linear. If the donor DNA is linear, it is preferred that the repeat sequences flanking the transposable element should not be at the termini of the linear fragment but should rather include some DNA upstream and downstream from the region flanked by the repeat sequences.

The transposable element between the OE termini can include any desired nucleotide sequence. The length of the transposable element between the termini should be at least about 50 base pairs, although smaller inserts may work. No upper limit to the insert size is known. However, it is known that a donor DNA portion of about 300 nucleotides in length can function well. By way of non-limiting examples, the transposable element can include a coding region that encodes a detectable or selectable protein, with or without associated regulatory elements such as promoter, terminator, or the like.

If the element includes such a detectable or selectable coding region without a promoter, it will be possible to identify and map promoters in the target DNA that are uncovered by transposition of the coding region into a position downstream thereof, followed by analysis of the nucleic acid sequences upstream from the transposition site.

Likewise, the element can include a primer binding site that can be transposed into the target DNA, to facilitate sequencing methods or other methods that rely upon the use of primers distributed throughout the target genetic material. Similarly, the method can be used to introduce a desired restriction enzyme site or polylinker, or a site suitable for another type of recombination, such as a cre-lox, into the target.

The invention can be better understood upon consideration of the following examples which are intended to be exemplary and not limiting on the invention.

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4.4.2. Generation of functional fusion protein products

The instant invention provides constructs and methods for the rapid and efficient generation of functional fusion protein products with either carboxy-terminal or amino-terminal fusions. Functional fusion proteins are those which retain some of the activity of the original domains, and/or those which have a newly created activity. Throughout this specification, reference is made to two types of fusions: carboxy terminal fusions and amino terminal fusions. In this text we use amino and carboxy terminal fusions to refer to the end of the domain inside of the Mu elements which is fused to the target molecule. Thus, carboxy terminal fusion elements are those with a protein domain inside of the Mu which extends out of the Mu element such that the exogenous protein is fused to the amino end of the endogenous protein. The amino terminal fusion elements are those that create fusions with a target gene extending into the element such that the exogenous protein is fused to the carboxy terminal of the endogenous protein.

An overview for generating both amino- and carboxy- terminal fusion proteins with the transposable elements is outlined. A domain of interest is inserted in one of two possible orientations into the end of a transposable element, such that a continuing open reading frame extends out through the element (for carboxy-terminal fusions) or in through the element (for amino-terminal fusions). Transposition into a target sequence allows random generation of hybrid proteins. Functional fusions can be selected or screened for.

4.4.2.1. Mini-Mu transposon elements & macro-Mu elements

The instant invention provides for mini-Mu transposon elements with convenient polylinker sites for inserting protein domains into the transposable element. The instant invention also provides for macro-Mu elements. The transposable elements of the instant invention have been designed to have a shorter transposon end which becomes incorporated into the fusion product while still retaining their high transposition frequencies. Unlike other elements used to date they can be used to make both amino and carboxy-terminal fusions since they have open reading frames extending in both directions. The examples below demonstrate the application of the teachings of the instant invention for using the new elements to create protein fusions with two fully active domains and their usefulness as a general tool for protein fusion. Other examples

demonstrate new features such as regulatable promoters incorporated into the transposable element, which can allow control of expression for promoterless domains or domains which may exhibit some degree of lethality to the cell. The constructs and methods described here allow high frequency random fusion of two domains at multiple sites so that the optimal fusion junctions can be selected.

4.4.2.2. The constructs

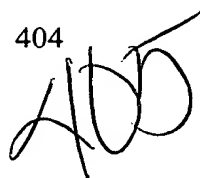
The constructs and teachings of the instant invention provide a powerful tool which will aid in the development of new enzymes for biocatalytic applications such as bioremediation and industrial biocatalysis, and for other industrial applications such as biosensors and strain development. Many different combinations of fusions between two domains can be generated rapidly and screened for activity. As an extension of protein evolution, this will be a powerful technique for production of novel chimeric proteins. In contrast to the difficulties inherent in a traditional rational protein design approach which employs traditional molecular biological techniques to generate fusion proteins, the instant invention provides for rapid and efficient "randomized experimentation" whereby the transposon fusion events are utilized to generate a panel of fusion proteins. From this panel of fusion proteins, selection for functionally expressed products, results in efficient screening for successful fusions.

While traditional experimentation with transposable elements resulted in the ablation of endogenous protein translation, and substitution of the translation and expression of the reporter gene for study of gene expression and regulation, the instant invention teaches modifications of these elements which allow for the generation of functional fusion proteins which can contain novel activities. The instant invention teaches for the first time the construction of transposable elements which can transpose exogenous DNA into target DNA in both orientations which can result in the functional expression of fusion proteins which have the exogenous protein linked either on the amino-end, or at the carboxy-end of the target protein.

The constructs and methods of the instant invention are not limited to the use of proteins with known crystal structures, but is capable of generating functional fusion proteins from

properties of the system are advantages which make Mu simple to use. Mu provides very convenient methods for isolating independent insertion events by transducing them to a new cell. Creation of insertions requires only a temperature shift. We have favored the Mu transposition system from the start because an untrained technician may be taught to reproducibly use the Mu system in just a few days. While the initial design and construction of a Mu system can be difficult, the finished system's simplicity lends itself to commercialization. There is much to be said for in vitro systems. The in vivo system of the instant invention was intended to be an additional protein engineering tool to enhance the methods of producing novel active protein fusions when used in conjunction with basic in vitro methods, or with additional novel in vitro methods of the instant invention. There has been no previous demonstration of the altered transcription through the end of a Mu element. Specifically, transcription through the end of the Mu element might reduce transposition frequencies. Surprisingly we have found that the transcription of the Protein A gene through the end of Mu did not alter transposition frequencies.

The instant invention encompasses both the amino and carboxy-terminal fusion elements because they are useful in different applications. The carboxy-terminal element is more useful for making fusions with a cDNA which has been cloned from a eukaryotic organism, especially if one wants to ultimately express it in another organism. Amino-terminal elements are more useful for some applications because they can only be activated by a fusion event if they are missing the start codon. The instant specification teaches new Mu elements with promoters for expression in the experimental design. One of the main commercial applications of the system is to generate new enzymes for medical or industrial uses both in vivo and in vitro. E. coli will likely be the production organism if there are no post-translational modification problems associated with the enzymes. But it is possible to use any Mu compatible strain as the production organism.



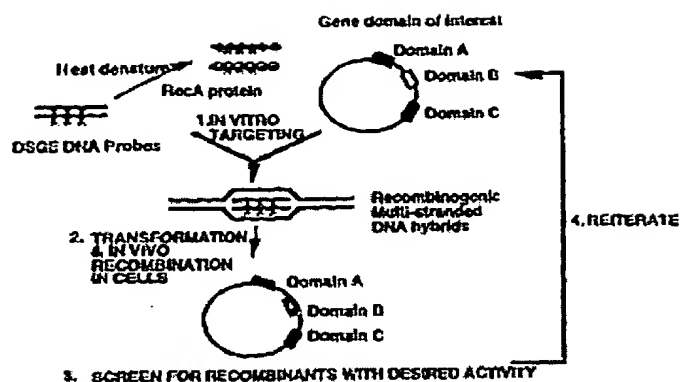
4.5. Additional Applications

It is envisioned that in addition to the uses specifically noted herein, other applications will be apparent to the skilled molecular biologist. In particular, methods for introducing desired mutations into prokaryotic or eukaryotic DNA are very desirable. For example, at present it is difficult to knock out a functional eukaryotic gene by homologous recombination with an inactive version of the gene that resides on a plasmid. The difficulty arises from the need to flank the gene on the plasmid with extensive upstream and downstream sequences. Using this system, however, an inactivating transposable element containing a selectable marker gene (e.g., neo) can be introduced in vitro into a plasmid that contains the gene that one desires to inactivate. After transposition, the products can be introduced into suitable host cells. Using standard selection means, one can recover only cell colonies that contain a plasmid having the transposable element. Such plasmids can be screened, for example by restriction analysis, to recover those that contain a disrupted gene. Such clones can then be introduced directly into eukaryotic cells for homologous recombination and selection using the same marker gene.

Also, one can use the system to readily insert a PCR-amplified DNA fragment into a vector, thus avoiding traditional cloning steps entirely. This can be accomplished by (1) providing suitable a pair of PCR primers containing OE termini adjacent to the sequence-specific parts of the primers, (2) performing standard PCR amplification of a desired nucleic acid fragment, (3) performing the in vitro transposition reaction of the present invention using the double-stranded products of PCR amplification as the donor DNA.

The invention is not intended to be limited to the foregoing examples, but to encompass all such modifications and variations as come within the scope of the appended claims.

5. Homologous Recombination



5.1. Homologous Recombination For The Generation Of Deletions And Insertions

The invention relates to compositions and methods of rapidly evolving specific protein domains using a library of nucleic acid filaments and a recombinase polypeptide or peptide. The invention relates to compositions and methods for targeting sequence modifications in one or more genes of a related family of genes using enhanced homologous recombination techniques. The invention also relates to compositions and methods for isolating and identifying novel members of homologous sequences families. These techniques may be used to create animal or plant models of disease as well as to identify new targets for drug or pathogen screening.

5.1.1. Evolution Of Genes

In nature, the evolution of genes and their encoded proteins occurs through an equilibrium between recombination or mutation and selection. While evolution in nature takes millions of years, in vitro methods and compositions have been developed to evolve proteins, with improved and novel functions, in a matter of hours to days.

5.1.1.1. Through Mutagenesis

Current in vitro gene evolution methods utilize repeated cycles of random mutagenesis or random nicking and mixing of related genes containing mutations in PCR-based random

recombination. These methods couple multiple rounds of in vitro mutagenesis with screening systems to produce and identify the desired mutants or recombinants (Stemmer 1994. *Nature* 370:389-391; Arnold 1996. *Chemical Engineering Science* 51:5091-5102). Research has shown, however, that the mutations of interest tend to occur in those regions or domains that are directly related to function (Chen and Arnold. 1993. *PNAS USA* 90:5618-5622).

However, these mutagenesis methods produce random mutations throughout the gene of interest which requires the need to screen large numbers of uninteresting or deleterious mutants. The labor-intensive and time consuming aspects of these methods are further complicated by the necessity of multiple rounds of subcloning and can be extremely challenging if the screening system is complex and does not utilize a selection system.

5.1.1.2. Homologous Recombination (HR)

Homologous recombination (HR) is defined as the exchange of homologous or similar DNA sequences between two DNA molecules. An essential feature of HR is that the enzymes responsible for the recombination event can pair any homologous sequences as substrates. The ability of HR to transfer genetic information between DNA molecules makes targeted homologous recombination a very powerful method in genetic engineering and gene manipulation. Both genetic and cytological studies have indicated that such a crossing-over process occurs between pairs of homologous chromosomes during meiosis in higher organisms.

5.1.1.3. Site-Specific Recombination

Alternatively, in site-specific recombination, exchange occurs at a specific site, as in the integration of phage A into the E coli chromosome and the excision of lambda DNA from it. Site-specific recombination involves specific inverted repeat sequences; e.g. the Cre-loxP and FLP-FRT systems. Within these sequences there is only a short stretch of homology necessary for the recombination event, but not sufficient for it. The enzymes involved in this event generally cannot recombine other pairs of homologous (or nonhomologous) sequences, but act specifically.

recombination required between resident and introduced sequences. These targeted recombination events can be used to correct mutations at known sites, replace genes or gene segments with defective ones, or introduce foreign genes into cells.

5.1.1.5.2. Frequency And Efficiency Of HR

HR can be used to add subtle mutations at known sites, replace wild type genes or gene segments or introduce completely foreign genes into cells. However, HR efficiency is very low in living cells and is dependent on several parameters, including the method of DNA delivery, how it is packaged, its size and conformation, DNA length and position of sequences homologous to the target, and the efficiency of hybridization and recombination at chromosomal sites. These variables severely limit the use of conventional HR approaches for gene evolution in cell based systems. (Kucherlapati et al. , 1984. PNAS; USA 81:3153-- 3157; Smithies et al. 1985. Nature 317:230-234; Song et al. 1987. PNAS USA 84:6820-6824; Doetschman et al. 1987. Nature 330:576-578; Kim and Smithies. 1988. Nuc. Acids. Res. 16:8887- 8903; Koller and Smithies. 1989. PNAS USA 86:8932-8935; Shesely et al. 1991. PNAS USA 88:4294- 4298; Kim et al. 1991. Gene 103:227-233).

5.1.1.5.2.1. Enhancement By The Presence Of Recombinase Activities

The frequency of HR is significantly enhanced by the presence of recombinase activities in cellular and cell free systems. Several proteins or purified extracts that promote HR (i.e., recombinase activity) have been identified in prokaryotes and eukaryotes (Cox and Lehman., 1987. Annu. Rev. Biochem. 56:229-262; Radding. 1982. Annual Review of Genetics 16:405-547; McCarthy et al. 1988. PNAS; USA 85:5854-5858). These recombinases promote one or more steps in the formation of homologously-paired intermediates, strand-exchange, and/or other steps.

Recent advances have resulted in techniques allowing enhanced homologous recombination (EHR) using recombinases such as recA and Rad51 and single-stranded nucleic acids that have sequence heterologies. This allows sequence modifications to be specifically targeted to virtually any genomic position. See for example, PCT US93/03868 and PCT US98/05223, both of which are expressly incorporated herein by reference.

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RecA protein binds cooperatively to any given sequence of single- stranded DNA with a stoichiometry of one RecA protein monomer for every three to four nucleotides in DNA (Cox and Lehman, 1987, *supra*). This forms unique right handed helical nucleoprotein filaments in which the DNA is extended by 1.5 times its usual length (Yu and Egelman 1992. *J. Mol. Biol.* 227:334-346). These nucleoprotein filaments, which are referred to as DNA probes, are crucial "homology search engines" which catalyze DNA pairing. Once the filament finds its homologous target gene sequence, the DNA probe strand invades the target and forms a hybrid DNA structure, referred to as a joint molecule or D-loop (DNA displacement loop) (McEntee et al. 1979. *PNAS USA* 76:2615-2619; Shibata et al. 1979. *PNAS USA* 76:1638-1642). The phosphate backbone of DNA inside the RecA nucleoprotein filaments is protected against digestion by phosphodiesterases and nucleases.

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5.1.1.5.3. Functional Genomics: The Correlation Of Genotype And Phenotype

One area of pressing interest in biology is within the area of "functional genomics", i.e. the correlation of genotype and phenotype. This requires animal systems, since phenotypic changes must be evaluated in vivo. Similarly, and related to this idea, is the elucidation and characterization of gene families, i.e. genes or proteins that are structurally related, i.e. they have sequence homologies between the members of the family. Since presumably many, if not most, disease states are caused by multiple gene interactions, the ability to evaluate interactions among genes, and particularly within or between gene families, at the phenotype level, would be extremely valuable.

5.2. Domain Specific Gene Evolution

5.2.1.1. Domain Specific Gene Evolution - Comprising Forming A Plurality Of Recombination Intermediates Comprising A Target Nucleic Acid Encoding An Amino Acid Sequence Of Interest, A Recombinase And A Plurality Of Targeting Polynucleotides

The present invention provides methods of domain specific gene evolution comprising forming a plurality of recombination intermediates comprising a target nucleic acid encoding an amino acid sequence of interest, a recombinase and a plurality of targeting polynucleotides. The targeting polynucleotides are substantially complementary to each other and each comprises a homology clamp that substantially correspond to or is substantially complementary to a predetermined sequence of the target nucleic acid and comprise random or degenerate sequences. The predetermined sequence encodes a domain of the amino acid sequence. The method further comprises contacting the intermediate with a recombination proficient cell, whereby a library of altered target nucleic acids are produced. The altered target nucleic acids are expressed in the cell to generate a pool of variant amino acid sequences. The method further comprises selecting and isolating a cell comprising an altered target nucleic acid that expressed a variant amino acid having a desired activity.

5.2.1.2. Comprising Forming A Recombination Intermediate Comprising A Target Nucleic Acid Encoding An Amino Acid Sequence Of Interest, A Recombinase And A Pair Of Targeting Poly Nucleotides

In another aspect of the invention, a method of domain specific gene evolution comprises forming a recombination intermediate comprising a target nucleic acid encoding an amino acid sequence of interest, a recombinase and a pair of targeting polynucleotides. The targeting polynucleotides are substantially complementary to each other and each comprises a homology clamp that substantially corresponds to or is substantially complementary to a predetermined sequence of the target nucleic acid. The predetermined sequence encodes a domain of the amino acid sequence. The method further comprises contacting the intermediate with a single-strand specific nuclease or junction-specific nuclease to form a nicked or open-ended target nucleic acid. The regions adjacent to the

hybridized region or junctions are susceptible to nucleases. The target nucleic acid is reassembled and recombined to produce a library of altered target nucleic acids. The target nucleic acids are expressed to generate a pool of variant amino acid sequences. The variant amino acid sequences are selected and characterized to identify an altered target nucleic acid encoding a variant amino acid sequence of interest.

In a further aspect, each method is repeated one or more times to further evolve a variant amino acid sequence having a desired activity. In yet another aspect, more than one domain or a protein is evolved simultaneously.

5.2.1.3. Compositions

It is an object of the present invention to provide compositions comprising at least one recombinase and at least two single-stranded targeting polynucleotides which are substantially complementary to each other and each having a consensus homology clamp for a gene family.

In an additional aspect, the invention provides compositions comprising at least one recombinase and a plurality of pairs of single stranded targeting polynucleotides, where the plurality of pairs comprises a set of degenerate probes encoding the consensus sequence.

In a further aspect, the invention provides kits comprising the compositions of the invention and at least one reagent.

5.2.1.4. Methods For Targeting A Sequence Modification In At Least One Member Of A Consensus Family Of Genes In A Cell By Homologous Recombination.

In an additional aspect, the invention provides methods for targeting a sequence modification in at least one member of a consensus family of genes in a cell by homologous recombination. The method comprises introducing into at least one cell at least one recombinase and at least two single-stranded targeting polynucleotides which are substantially complementary to each other and each having a consensus homology clamp for the family. The method can additionally comprise identifying a target cell having a targeted sequence modification.

In a further aspect, the invention provides methods of making a non- human organism with a targeted sequence modification in at least one member of a gene family. The method comprises introducing into a cell at least one recombinase and at least two single-stranded targeting polynucleotides which are substantially complementary to each other and each having a consensus homology clamp for said family. The cell is then subjected to conditions that result in the formation of an animal, and the animal has at least one modification in at least one member of a consensus family of genes.

In a further aspect, the invention provides non-human organisms containing a sequence modification in an endogenous consensus functional domain of a gene member of a gene family.

5.2.1.5. Methods Of Isolating A Member Of A Gene Family Comprising A Protein Consensus Sequence

In an additional aspect, the invention provides methods of isolating a member of a gene family comprising a protein consensus sequence. The method comprises adding to a complex mixture of nucleic acids at least one recombinase and at least two single-stranded targeting polynucleotides which are substantially complementary to each other and each having a consensus homology clamp for said family. At least one of the targeting polynucleotides comprises a purification tag. The method is done under conditions whereby the targeting polynucleotides form a complex with the member, and the family member is isolated using said purification tag. The complex nucleic acid mixture may be a cDNA library, a cell, RNA or a restriction endonucleases genomic digest.

5.3. Targeting A Predetermined Nucleic Acid Sequence That Encodes A Specific Protein Domain, To Make A Plurality Of Targeted Sequence Modifications

The present invention provides methods and compositions for domain specific gene evolution. In one aspect of the invention, the method comprises targeting a predetermined nucleic acid sequence that encodes a specific protein domain, to make a plurality of targeted sequence modifications. That is, by targeting the recombinogenic probes of the

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invention to particular protein domains, gene evolution and selection are targeted to specific domains known or believed to harbor specific activities or functions. These methods create maximal diversity in specific domains of interest, thereby, decreasing the size of the library of mutations that are to be screened and increasing the probability of finding a gene with improved or desired attributes. Therefore, the libraries of the present invention are enriched for advantageous or interesting mutations or recombinant sequence(s).

5.3.1. Combining A Plurality Of Pairs Of Single-Stranded Targeting Poly Nucleotides, A Predetermined Target Nucleic Acid, And A Recombinase To Form A Polynucleotide:Target Nucleic Acid Complex

5.3.2. Domain Specific DNA Nicking

inactivated and the complex dissociated. The nicked target nucleic acid is reassembled and recombined by PCR to produce a library of nucleic acids with preferential modifications in the nicked regions. The library of modified nucleic acids can be introduced into a host cell and expressed. Cells are selected and isolated that comprise a modified nucleic acid that encodes a polypeptide having a desired property. This process is repeated iteratively to further evolve the predetermined targeted domain of interest.

In each of the methods described above, single domains and optionally multiple domains are targeted. The methods and compositions described above are optionally used in combination for domain specific gene evolution. For example, individual or multiple rounds of domain specific DNA nicking are followed or interspersed with one or more rounds of domain specific evolution employing a plurality of targeting polynucleotides described above.

The methods of the present invention also avoid multiple subcloning steps. This is particularly relevant when large complex vectors such as lambda, BACS, PACS, YACS, MACS and other genomic DNAs are used and where multiple subcloning steps make mutagenesis and shuffling of unique sites in large vectors particularly tedious and time consuming.

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oxidants, to proteases, etc.); altered specificity (for example, in the case of enzymes); altered binding; modified activity and other desirable properties, such as, altered immunogenicity.

5.3.4. Use Of Homology Motif Tags (HMTs) In Targeted Homologous Recombination To Elucidate Disease Mechanisms And To Identify Disease Targets Contained Within Gene Families

Interestingly, while a wide variety of gene families are known, the majority of drug targets come from only four of these gene families. These are the G-protein coupled or seven-transmembrane domain receptors, nuclear (hormone) receptors, ion channels, esterases. Other important gene families are enzymes, including recombinases. Of the top 100 pharmaceutical drugs, 18 bind to seven- transmembrane receptors, 10 to nuclear receptors and 16 to ion channels.

sequences (e.g., structural genes, regulatory sequences including promoters and enhancers, recombinatorial hotspots, repeat sequences, integrated proviral sequences, hairpins, palindromes), episomal or extrachromosomal sequences (e.g., replicable plasmids or viral replication intermediates) including chloroplast and mitochondrial DNA sequences. By "predetermined" or "pre-selected" it is meant that the consensus functional domain target sequence may be selected at the discretion of the practitioner on the basis of known or predicted sequence information, and is not constrained to specific sites recognized by certain site-specific recombinases (e.g., FLP recombinase or CRE recombinase). In some embodiments, the predetermined endogenous DNA target sequence will be other than a naturally occurring germline DNA sequence (e.g., a transgene, parasitic, mycoplasmal or viral sequence).

5.3.4.1.1. Gene Family Is The G-Protein Coupled Receptor Family

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hormone (gonadotropin releasing hormone, thyrotropin-releasing hormone, growth hormone secretagogue), melatonin, viral proteins, MHC receptor, Mas proto-oncogene, EBV-induced and glucocorticoid induced; the class B secretin second subfamily, including calcitonin, corticotropin releasing factor, gastric inhibitory peptide, glucagon, growth hormone releasing hormone, parathyroid hormone, secretin, vasoactive intestinal polypeptide, and diuretic hormone; the class C metabotropic glutamate third subfamily, including metabotropic glutamate and extracellular calcium-sensing agents; and the class D pheromone fourth subfamily. Because of the large number of family members, these large classes of GPCRs can be further subdivided into subfamilies. Examples of these subfamilies are calcitonin, glucagon, vasoactive and parathyroid are from class B; and acetylcholine, histamine angiotensin, alpha2- and beta-adrenergic are from class A. From each subfamily small protein consensus sequences can be derived from sequence alignments. For example, there are 6 motifs for the metabotropic glutamate like GPCRs derived from the indicated number of family members. Using the protein consensus sequence, degenerate nucleic acid probes are made to encode the protein consensus sequence, as is well known in the art. The protein sequence is encoded by DNA triplets which are deduced using standard tables. In some cases additional degeneracy is used to enable production in one oligonucleotide synthesis. In many cases motifs were chosen to minimize degeneracy. Amplification of neighboring sequences can utilize two motifs as indicated by faithful or error prone amplification. Alternatively outside sequences can be used as is indicated using vector sequence. In addition degenerate oligos can be synthesized and used directly in the procedure without amplification. Double stranded (ds) DNA probes are denatured and coated with RecA or another recombinase such as Rad51. This material can be used to bind to and allow capture of specific clones from cDNA or genomic libraries. Alternatively this material can be introduced into cells producing transgenic cells or animals with alterations in related family members.

5.3.4.1.1.2. Second Subfamily Encoding Receptors That Bind Peptide Hormones That Do Not Show Sequence Similarity To The First R7G Subfamily

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calcium pathway. However, they are structurally similar; like classical R7G proteins they putatively contain seven transmembrane regions, a glycosylated extracellular N-terminus and a cytoplasmic C-terminus. Known receptors in this subfamily are encoded on multiple exons, and several of these genes are alternatively spliced to yield functionally distinct products. The N-terminus contains five conserved cysteine residues putatively important in disulfide bonds. Known G-protein coupled receptors in this subfamily are listed above.

5.3.4.1.1.3. Third Subfamily Encoding Receptors That Bind Glutamate And Calcium But Do Not Show Sequence Similarity To Either Of The Other Subfamilies

5.3.4.1.2. Gene Family Is The bZIP Transcription Factor Family

In a preferred embodiment, the gene family is involved in DNA mismatch repair, such as mutL, hexB and PMS1. Members of this family include, but are not limited to, MLH1, PMS1, PMS2, HexB and MutL. The protein consensus sequence is G-F-R-G-E-A-L.

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apoptosis proteins, such as Bcl-2 and Bcl-x(L). Known Bcl-2 proteins include, but are not limited to, Bcl-2, Bcl-x(L), Bcl-W, Bcl-x(S), Bad, Bax, and Bak.

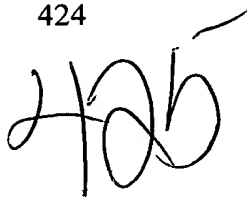
5.3.4.1.5. Gene Family Is The Site-Specific Recombinase Family

In a preferred embodiment, the gene family is the site-specific recombinase family. Site-specific recombination plays an important role in DNA rearrangement in prokaryotic organisms. Two types of site-specific recombination are known to occur: a) recombination between inverted repeats resulting in the reversal of a DNA segment; and b) recombination between repeat sequences on two DNA molecules resulting in their cointegration, or between repeats on one DNA molecule resulting the excision of a DNA fragment. Site-specific recombination is characterized by a strand exchange mechanism that requires no DNA synthesis or high energy cofactor; the phosphodiester bond energy is conserved in a phospho-protein linkage during strand cleavage and re- ligation.

Two unrelated families of recombinases are currently known. The first, called the "phage integrase" family, groups a number of bacterial, phage and yeast plasmid enzymes. The second, called the "resolvase" family, groups enzymes which share the following structural characteristics: an N-terminal catalytic and dimerization domain that contains a conserved serine residue involved in the transient covalent attachment to DNA, and a C-terminal helix-turn-helix DNA- binding domain.

5.3.4.1.6. Gene Family Is The Single-Stranded Binding Protein Family

In a preferred embodiment, the gene family is the single-stranded binding protein family. The E coli single-stranded binding protein (ssb), also known as the helix- destabilizing protein, is a protein of 177 amino acids. It binds tightly as a homotetramer to a single-stranded DNA (ss-DNA) and plays an important role in DNA replication, recombination and repair. Members of the ssb family include, but are not limited to, E. coli ssb and eukaryotic RPA proteins.



545; Kolodner et al., (1987) Proc. Natl. Acad. Sci. USA 84: 5560; Sugino et al., (1985) Proc. Natl. Acad. Sci. USA 85: 3683; Halbrook et al., (1989) J. Biol. Chem. 264: 21403; Eisen et al., (1988) Proc. Natl. Acad. Sci. USA 85: 7481; McCarthy et al., (1988) Proc. Natl. Acad. Sci. US 85: 5854; Lowenhaupt et al., (1989) J. Biol. Chem 264: 20568, which are incorporated herein by reference. Examples of such recombinase proteins include, for example but not limited to: RecA, RecA803, uvsX, and other RecA mutants and RecA-like recombinases (Roca, A. 1. (1990) Crit. Rev. Biochem. Molec. Biol. 25: 415), sep1 (Kolodner et al. (1987) Proc. Natl. Acad. Sci. (U.S.6.1 B4:5560; Tishkoff et al. Molec. Cell. Biol. 11:2593), RuvC (Dunderdale et al. (1991) Nature 354: 506), DST2, KEM1, XRN 1 (Dykstra et al. (1991) Molec. Cell. Biol. 11:2583), STPalph/DST1 (Clark et al. (1991) Molec. Cell. Biol. 11:2576), HPP-1 (Moore et al. (1991) Proc. Natl. Acad. Sci. (U.S.A.1 B8:9067), other target recombinases (Bishop et al. (1992) Cell 69 439; Shinohara et al. (1992) Cell 69 457); incorporated herein by reference. RecA may be purified from E coli strains, such as E coli strains JC 12772 and JC1 5369 (available from A.J. Clark and M. Madiraju, University of California-Berkeley, or purchased commercially). These strains contain the RecA coding sequences on a "runaway" replicating plasmid vector present at a high copy numbers per cell. The RecA803 protein is a high-activity mutant of wild- type RecA. The art teaches several examples of recombinase proteins, for example, from Drosophila, yeast, plant, human, and non-human mammalian cells, including proteins with biological properties similar to RecA (i.e., RecA-like recombinases), such as Rad51, Rad55, Rad57, dmcl from mammals and yeast. In addition, the recombinase may actually be a complex of proteins, i.e. a "recombinosome". In addition, included within the definition of a recombinase are portions or fragments of recombinases which retain recombinase biological activity, as well as variants or mutants of wild- type recombinases which retain biological activity, such as the E. coli RecA803 mutant with enhanced recombinase activity.

5.3.6.1. RecA or rad51

In a preferred embodiment, RecA or rad51 is used. For example, RecA protein is typically obtained from bacterial strains that overproduce the protein: wild-type E coli RecA protein and mutant RecA803 protein may be purified from such strains. Alternatively, RecA protein can also be purchased from, for example, Pharmacia (Piscataway, NJ) or Boehringer Mannheim (Indianapolis, Indiana).

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The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any

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introduction of the target nucleic acid results in expression of the encoded protein. The transcription control elements include a promoter, such as, a constitutive or inducible promoter. When the host cell of interest is a eukaryotic cell, enhancer elements are optionally employed. In a preferred embodiment the target nucleic acid is an extrachromosomal vector such as a plasmid. In other embodiments, the target nucleic acid is a viral vector, such as, a retrovirus, a phage, a BAC, PAC, YAC, MAC or other types of genomic and chromosomal DNA.

The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

5.3.6.1.1.2. The Target Nucleic Acid Comprises A Nucleic Acid Encoding A Protein Domain

The methods of the invention are used for alteration and evolution of protein domains; that is, in a preferred embodiment, the target nucleic acid comprises a nucleic acid encoding a protein domain. By "protein domain" and grammatical equivalents as used herein are meant a region of a protein that provides a specific structural and/or functional characteristic. Accordingly, a protein domain is an enzymatic active site, a ligand binding site, an allosteric effector region, an epitope, a region of a protein that is modified, such as, by addition of a carbohydrate, phosphate or lipid. A domain also relates to the hydrophobicity or hydrophilicity of a region and, therefore, also includes extracellular, intracellular, and transmembrane domains. Cell targeting sequences, such as, a signal peptide, nuclear localization sequence, mitochondrial localization sequences, etc. that direct proteins to either an extracellular or subcellular locale are domains. Additional domains include regions of proteins that interact with other proteins or nucleic acids, for example, include multimerization sequences, zinc- finger motifs, and the like. In another aspect, a protein domain is a region encoded by an exon.

Targeting polynucleotides have at least one sequence that substantially corresponds to, or is substantially complementary to, a target nucleic acid; in a preferred embodiment, it corresponds or complements a nucleic acid encoding a protein domain. By "corresponds

may be used to select appropriate hybridization conditions (see, Maniatis et al., Molecular Cloning: A Laboratory Manual (1989), 2nd Ed., Cold Spring Harbor, N.Y. and Berger and Kimmel, Methods in Enzymology. Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, CA.), which are incorporated herein by reference. Methods for hybridizing a targeting polynucleotide to a discrete chromosomal location in intact nuclei are known in the art, see for example WO 93/05177 and Kowalczykowski and Zarling (1994) in Gene Targeting, Ed. Manuel Vega.

In targeting polynucleotides, domain homology clamps are typically located at or near the 5' or 3' end, preferably domain homology clamps are internal or located at each end of the polynucleotide (Berinstein et al. (1992) Molec. Cell. Biol. 12: 360, which is incorporated herein by reference). Without wishing to be bound by any particular theory, it is believed that the addition of recombinases permits efficient gene targeting with targeting polynucleotides having short (i. e., about 10 to 1000 basepair long) segments of homology, as well as with targeting polynucleotides having longer segments of homology.

5.3.9. Targeting Polynucleotides

5.3.9.1. Targeting Polynucleotides That Have Domain Homology Clamps That Are Highly Homologous To The Predetermined Target Endogenous Domain Functional Domain Nucleic Acid Sequence

Therefore, it is preferred that targeting polynucleotides of the invention have domain homology clamps that are highly homologous to the predetermined target endogenous domain functional domain nucleic acid sequence(s). Typically, targeting polynucleotides of the invention have at least one domain homology clamp that is at least about 18 to 35 nucleotides long, and it is preferable that domain homology clamps are at least about 20 to 100 nucleotides long, and more preferably at least about 100-500 nucleotides long, although the degree of sequence homology between the domain homology clamp and the targeted sequence and the base composition of the targeted sequence will determine the optimal and minimal clamp lengths (e.g., G-C rich sequences are typically more thermodynamically stable and will generally require shorter clamp length). Therefore, both domain homology clamp length and the degree of sequence homology can only be determined with reference to a particular predetermined sequence, but domain homology clamps generally must be at least about 10 nucleotides long and must also substantially

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correspond or be substantially complementary to a predetermined target sequence. Preferably, a homology clamp is at least about 10, and preferably at least about 50 nucleotides long and is substantially identical to or complementary to a predetermined target sequence. Without wishing to be bound by a particular theory, it is believed that the addition of recombinases to a targeting polynucleotide enhances the efficiency of homologous recombination between homologous, nonisogenic sequences (e.g., between an exon 2 sequence of an albumin gene of a Balb/c mouse and a homologous albumin gene exon 2 sequence of a C57/BL6 mouse), as well as between isogenic sequences.

5.3.9.2. Targeting Polynucleotides Comprising A Plurality Of Targeting Polynucleotides Comprising At Least One Shared Homology Clamp And A Degenerate Sequence

In one aspect of the invention, the targeting polynucleotides comprise a plurality of targeting polynucleotides comprising at least one shared homology clamp and a degenerate sequence. By “plurality” herein is meant more than one. The targeting polynucleotides find use in the mutagenesis and evolution of a target nucleic acid sequence that encodes specific protein domain by insertion, deletion and/or substitution of the nucleic acid sequence encoding the domain. In one embodiment the degenerate sequence is completely randomized, representing all possible combinations of nucleotides. In another embodiment, the degenerate sequence is biased, for example, to eliminate sequences encoding for transcriptional or translational stop signals. In another embodiment, the degenerate sequence is biased, to represent the codon bias of a host cell or class of organisms. The degenerate sequence is optionally biased to randomize specific sequence while maintaining other sequences constant. The length of the degenerate sequence is determined by the practitioner and is based on the desired number of nucleotides within the predetermined sequence to be modified.

5.3.9.3. Targeting Polynucleotides Substantially Identical To The Predetermined Target Sequence

In an alternative embodiment, the targeting polynucleotides are substantially identical to the predetermined target sequence. In the presence of a recombinase, the targeting polynucleotides form complexes with a predetermined target sequence of a target nucleic acid. As a part of the complex, the predetermined target sequence is resistant to nuclease

digestion. The regions flanking the polynucleotide:target complex are susceptible to single-strand specific exonucleases. Accordingly, to effect domain specific evolution, these regions are nicked and the resultant fragments are reassembled and recombined by PCR as described below and by Stemmer et al. Nature. 370:389-391 and Stemmer et al. PNAS USA 91:10747-10751, hereby incorporated by reference.

The formation of heteroduplex joints is not a stringent process; genetic evidence supports the view that the classical phenomena of meiotic gene conversion and aberrant meiotic segregation results in part from the inclusion of mismatched base pairs in heteroduplex joints, and the subsequent correction of some of these mismatched base pairs before replication. Observations on RecA protein have provided information on parameters that affect the discrimination of relatedness from perfect or near-perfect homology and that affect the inclusion of mismatched base pairs in heteroduplex joints. The ability of RecA protein to drive strand exchange past all single base-pair mismatches and to form extensively mismatched joints in superhelical DNA reflect its role in recombination and gene conversion. This error-prone process may also be related to its role in mutagenesis. RecA-mediated pairing reactions involving DNA of X 1 74 and G4, which are about 70 percent homologous, have yielded homologous recombinants (Cunningham et al. (1981) Cell 24: 213), although RecA preferentially forms homologous joints between highly homologous sequences, and is implicated as mediating a homology search process between an invading DNA strand and a recipient DNA strand, producing relatively stable heteroduplexes at regions of high homology. Accordingly, it is the fact that recombinases can drive the homologous recombination reaction between strands which are significantly, but not perfectly, homologous, which allows gene conversion and the modification of target sequences. Thus, targeting polynucleotides may be used to introduce nucleotide substitutions, insertions and deletions into an endogenous functional domain nucleic acid sequence, and thus the corresponding amino acid substitutions, insertions and deletions in proteins expressed from the endogenous domain functional domain nucleic acid sequence. By "endogenous" in this context herein is meant the naturally occurring sequence, i.e. sequences or substances originating from within a cell or organism. Similarly, "exogenous" refers to sequences or substances originating outside the cell or organism.

the strand exchange phase. The double D-loop permits isolation of stable multistranded DNA recombination intermediates.

In addition, when the targeting polynucleotides are used to generate insertions or deletions in an endogenous nucleic acid sequence, as is described herein, the use of two complementary single-stranded targeting polynucleotides allows the use of internal homology clamps. The use of internal homology clamps allows the formation of stable deproteinized cssDNA:probe target hybrids with homologous DNA sequences containing either relatively small or large insertions and deletions within a homologous DNA target. Without being bound by theory, it appears that these probe:target hybrids, with heterologous inserts in the cssDNA probe, are stabilized by the re-annealing of cssDNA probes to each other within the double-D-loop hybrid, forming a novel DNA structure with an internal homology clamp. Similarly stable double-D-loop hybrids formed at internal sites with heterologous inserts in the linear DNA targets (with respect to the cssDNA probe) are equally stable. Because cssDNA probes are kinetically trapped within the duplex target, the multi-stranded DNA intermediates of homologous DNA pairing are stabilized and strand exchange is facilitated.

5.3.10. Length Of The Internal Homology Clamp (i. e. The Length Of The Insertion Or Deletion)

In a preferred embodiment, the length of the internal homology clamp (i. e. the length of the insertion or deletion) is from about 1 to 50% of the total length of the targeting polynucleotide, with from about 1 to about 20% being preferred and from about 1 to about 10% being especially preferred, although in some cases the length of the deletion or insertion may be significantly larger. As for the domain homology clamps, the complementarity within the internal homology clamp need not be perfect.

A targeting polynucleotide used in a method of the invention typically is a single-stranded nucleic acid, usually a DNA strand, or derived by denaturation of a duplex DNA, which is complementary to one (or both) strand(s) of the target duplex nucleic acid. Thus, one of the complementary single stranded targeting polynucleotides is complementary to one strand of the endogenous target sequence (i.e. Watson) and the other complementary single stranded targeting polynucleotide is complementary to the other strand of the endogenous target sequence (i.e. Crick). The domain homology clamp sequence preferably contains at

5.3.12.1.1.1. Disruption By Either The Substitution, Insertion, Deletion Or Frame Shifting Of Nucleotides

The endogenous target sequence, generally nucleic acid encoding a domain, may be disrupted in a variety of ways. The term "disrupt" as used herein comprises a change in the coding or non-coding sequence of an endogenous nucleic acid. In one preferred embodiment, a disrupted gene will no longer produce a functional gene product. In another preferred embodiment, a disrupted gene produces a variant gene product. Generally, disruption may occur by either the substitution, insertion, deletion or frame shifting of nucleotides.

5.3.12.1.1.2. Disruption By Amino Acid Substitutions

In one embodiment, amino acid substitutions are made. This can be the result of either the incorporation of a non-naturally occurring domain sequence into a target, or of more specific changes to a particular sequence outside of the domain sequence.

5.3.12.1.1.3. Disruption By An Insertion Sequence

In one embodiment, the endogenous sequence is disrupted by an insertion sequence. The term "insertion sequence" as used herein means one or more nucleotides which are inserted into an endogenous gene to disrupt it. In general, insertion sequences can be as short as 1 nucleotide or as long as a gene, as outlined herein. For non-gene insertion sequences, the sequences are at least 1 nucleotide, with from about 1 to about 50 nucleotides being preferred, and from about 10 to 25 nucleotides being particularly preferred. An insertion sequence may comprise a polylinker sequence, with from about 1 to about 50 nucleotides being preferred, and from about 10 to 25 nucleotides being particularly preferred. Insertion sequence may be a PCR tag used for identification of the first gene. In a preferred embodiment, an insertion sequence comprises a gene which not only disrupts the endogenous gene, thus preventing its expression, but also can result in the expression of a new gene product. Thus, in a preferred embodiment, the disruption of an endogenous gene by an insertion sequence gene is done in such a manner to allow the transcription and translation of the insertion gene. An insertion sequence that encodes a gene may range from about 50 bp to 5000 bp of cDNA or about 5000 bp to 50000 bp of genomic DNA. As will be appreciated by those in the art, this can be done in a variety of ways. In a preferred embodiment, the insertion gene is targeted to the endogenous gene in

employed as cell-uptake components. Nucleases, DNA damaging chemicals, UV radiation or gamma- radiation may also be used.

In addition to cell-uptake components, targeting components such as nuclear localization signals may be used, as is known in the art. See for example Kido et al., Exper. Cell Res. 198:107-114 (1992), hereby expressly incorporated by reference.

Typically, a targeting polynucleotide of the invention is coated with at least one recombinase and is conjugated to a cell-uptake component, and the resulting cell targeting complex is contacted with a target cell under uptake conditions (e.g., physiological conditions) so that the targeting polynucleotide and the recombinase(s) are internalized in the target cell. A targeting polynucleotide may be contacted simultaneously or sequentially with a cell-uptake component and also with a recombinase; preferably the targeting polynucleotide is contacted first with a recombinase, or with a mixture comprising both a cell-uptake component and a recombinase under conditions whereby, on average, at least about one molecule of recombinase is noncovalently attached per targeting polynucleotide molecule and at least about one cell-uptake component also is noncovalently attached. Most preferably, coating of both recombinase and cell-uptake component saturates essentially all of the available binding sites on the targeting polynucleotide. A targeting polynucleotide may be preferentially coated with a cell-uptake component so that the resultant targeting complex comprises, on a molar basis, more cell-uptake component than recombinase(s). Alternatively, a targeting polynucleotide may be preferentially coated with recombinase(s) so that the resultant targeting complex comprises, on a molar basis, more recombinase(s) than cell- uptake component.

Cell-uptake components are included with recombinase-coated targeting polynucleotides of the invention to enhance the uptake of the recombinase-coated targeting polynucleotide(s) into cells, particularly for in vivo gene targeting applications, such as gene therapy to treat genetic diseases, including neoplasia, and targeted homologous recombination to treat viral infections wherein a viral sequence (e.g., an integrated hepatitis B virus (HBV) genome or genome fragment) may be targeted by homologous sequence targeting and inactivated. Alternatively, a targeting polynucleotide may be coated with the cell-uptake component and targeted to cells with a contemporaneous or

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5.3.13.2.2. Biased Library

In a preferred embodiment, the library is biased. That is, some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in a preferred embodiment, the nucleotides or amino acid residues are randomized within a defined class, for example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either small or large) residues, towards the creation of cysteines, for cross-linking, prolines for SH-3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, etc., or to purines, etc.

As will be appreciated by those in the art, the introduction of a pool of variant targeting polynucleotides (in combination with recombinase) to a target sequence, in vitro to an extrachromosomal sequence, can result in a large number of homologous recombination reactions occurring over time. That is, any number of homologous recombination reactions can occur on a single target sequence, to generate a wide variety of single and multiple mismatches within a single target sequence, and a library of such variant target sequences, most of which will contain mismatches and be different from other members of the library. This thus works to generate a library of mismatches.

5.3.13.2.2.1. Generating A Large Number Of Different Variants Within A Particular Region Of A Sequence, Similar To Cassette Mutagenesis But Not Limited By Sequence Length

In a preferred embodiment, the variant targeting polynucleotides are made to a particular region or domain of a sequence (i.e. a nucleotide sequence that encodes a particular protein domain). For example, it may be desirable to generate a library of all possible variants of a binding domain of a protein, without affecting a different biologically functional domain, etc. Thus, the methods of the present invention find particular use in generating a large number of different variants within a particular region of a sequence, similar to cassette mutagenesis but not limited by sequence length. This idea is sometimes referred to herein as "domain specific gene evolution". In addition, two or more regions may also be altered simultaneously using these techniques; thus "single domain" and "multi- domain" shuffling can be done. Suitable domains include, but are not limited to, kinase domains, nucleotide-binding sites, DNA binding sites, signaling domains, receptor

example an antibody phage library, one can change the sequences in the CDR regions of both the heavy and light chains. Different permutations and combinations of CDRs can be changed and evolved to engineer antibody-phage libraries.

5.3.14.2. Target Sequence - A Single-Chain Fv Framework For Any Number Of Specific Antigens

5.3.14.3. Target Sequence - An Antibody-Phage Fusion

5.3.14.4. Target Sequence - The Coding Sequence For beta-Lactamase

Thus, the methods of the invention may be used to create superior recombinant reporter genes such as lacZ and green fluorescent protein (GFP); superior antibiotic and drug resistance genes; superior recombinase genes; superior recombinant vectors; and other superior recombinant genes and proteins, including immunoglobulins, vaccines or other proteins with therapeutic value. For example, targeting polynucleotides containing any number of alterations may be made to one or more functional or structural domains of a protein, and then the products of homologous recombination evaluated.

Once made and administered to target cells, the target cells may be screened to identify a cell that contains the targeted sequence modification. This will be done in any number of

Jurkat T cells, NIH 3T3 cells, CHO, Cos, etc. See the ATCC cell line catalog, hereby expressly incorporated by reference.

For making transgenic non-human animals (which include homologously targeted non-human animals) embryonal stem cells (ES cells), donor cells for nuclear transfer and fertilized zygotes are preferred. In a preferred embodiment, embryonal stem cells are used. Murine ES cells, such as AB-1 line grown on mitotically inactive SNL76/7 cell feeder layers (McMahon and Bradley, Cell 62: 1073-1085 (1990)) essentially as described (Robertson, E.J. (1987) in Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. E.J. Robertson, ed. (Oxford: IRL Press), p. 71-112; Zijlstra et al., Nature 342:435-438 (1989); and Schwartzberg et al., Science 246:799-803 (1989), each of which is incorporated herein by reference) may be used for homologous gene targeting. Other suitable ES lines include, but are not limited to, the E14 line (Hooper et al. (1987) Nature 326: 292-295), the D3 line (Doetschman et al. (1985) J. Embryol. Exp. Morph. 87: 21-45), and the CCE line (Robertson et al. (1986) Nature 323: 445-448). The success of generating a mouse line from ES cells bearing a specific targeted mutation depends on the pluripotency of the ES cells (i.e., their ability, once injected into a host blastocyst, to participate in embryogenesis and contribute to the germ cells of the resulting animal).

The pluripotency of any given ES cell line can vary with time in culture and the care with which it has been handled. The only definitive assay for pluripotency is to determine whether the specific population of ES cells to be used for targeting can give rise to chimeras capable of germline transmission of the ES genome. For this reason, prior to gene targeting, a portion of the parental population of AB-1 cells is injected into C57B1/6J blastocysts to ascertain whether the cells are capable of generating chimeric mice with extensive ES cell contribution and whether the majority of these chimeras can transmit the ES genome to progeny.

5.3.22. Non-Human Zygotes Are Used

In a preferred embodiment, non-human zygotes are used, for example to make transgenic animals, using techniques known in the art (see U.S. Patent No. 4,873,191; Brinster et al., PNAS 86:7007 (1989); Susulic et al., J. Biol. Chem. 270:29483 (1995), and Cavard et al., Nucleic Acids Res. 16:2099 (1988), hereby incorporated by reference). Preferred zygotes

pigmentosa allele. As used herein, a disease allele encompasses both alleles associated with human diseases and alleles associated with recognized veterinary diseases. For example, the deltaF508 CFTR allele in a human disease allele which is associated with cystic fibrosis in North Americans.

Once made and administered to target cells, new domains of genes may be isolated as outlined herein.

Alternatively, the target cells may be screened to identify a cell that contains the targeted functional domain sequence modification. This will be done in any number of ways, and will depend on the target domain and targeting polynucleotides as will be appreciated by those in the art. The screen may be based on phenotypic, biochemical, genotypic, or other functional changes, depending on the target sequence. For example, IgE levels may be evaluated for inflammation or asthma; vascular tone or blood pressure can be evaluated for hypertension, behavior screens can be done for neurologic effects, lipoprotein profiles can be screened for cardiovascular effects; secreted molecules can be evaluated for endocrine processes; CBCs can be done for hematology studies, etc. In an additional embodiment, as will be appreciated by those in the art, selectable markers or marker sequences may be included in the targeting polynucleotides to facilitate later identification.

The broad scope of this invention is best understood with reference to the following examples, which are not intended to limit the invention in any manner. All patents, patent applications, and publications cited herein are expressly incorporated by reference in their entirety.

5.5.2.2.2. Controlling Expression Of A Group Of Unlinked Genes That Aid In Recovery Of Cells After Exposure To DNA Damaging Agents

The recA gene performs another equally important role in cell metabolism by controlling expression of a group of unlinked genes that aid in recovery of cells after exposure to DNA-damaging agents. This response, termed the SOS response, involves genes that participate in repair of DNA damage, mutagenesis, and coordination of cell division events.

5.5.2.2.3. Characterization

The purified RecA protein is a single polypeptide ranging in weight from about 37,000 to about 42,000. Although there is some variation in the sequences between bacterial strains, the recA proteins from a variety of bacteria in general have been isolated and characterized by interspecies complementation and assays utilizing comparisons with isolated, characterized proteins.

The present method and the linear fragments for use in the present method are not limited to organisms such as *E. coli*. The recA gene is found in a variety of bacteria including both gram negative and gram positive organisms. The recA gene has been isolated or identified and characterized in several organisms. It is possible to prepare a genomic library from any bacterial species and to isolate a clone containing a sequence homologous to a characterized recA gene by interspecific complementation using one of the available DNA clones containing a recA sequence or cross-reactivity with antisera to RecA proteins from a well-characterized organism such as *E. coli*.

RecA⁺ and RecA⁻ strains are available from a variety of sources. For example, cloning and characterization of recA genes and recA proteins from *Proteus vulgaris*, *Erwinia carotovoria*, *Shigella flexneria* and *Escherichia coli* are described by S. L. Keener, et al., in *J. Bacter.* 160(1), 153-160 (1984). The RecA proteins produced by these organisms were demonstrated to be highly conserved among the species. In fact, the protein produced by one species could be introduced into another species where it complemented repair and regulatory defects of recA mutations. Other bacterial recA genes and gene products have been described by C.A. Miles, et al, in *Mol. Gen.Genet.* 204,161-165 (1986) (*Agrobacterium tumefaciens* C58), I. Goldberg et al, *J. Bacteriol.* 165(3), 715-722(1986),

(*Vibrio cholera*), M. Better et al, J. Bacteriol. 155(1), 311-316 (1983), (*Rhizobium meliloti*), T.A. Kokjohn et al, J. Bacteriol. 163(2), 568-572 (1985), (*Pseudomonas aeruginosa*), and C.M. Lovett, Jr. et al, J. Bio.Chem. 260(6), 3305-3313 (1985) (*Bacillus subtilis*). These articles detail the isolation and characterization of gene libraries and the proteins encoded by the *recA* genes using techniques known to those skilled in the art including construction of gene libraries, identification of homologous genes using hybridization to probes from other more well characterized species such as *E. coli*, isolation and characterization of RecA proteins using antisera to RecA proteins from *E. coli*, and interspecies complementation of deficient strains of *E. coli* using gene segments from the libraries. The isolated proteins were useful for in vitro complementation studies. RecA deficient strains and RecA clones are available from many of the laboratories cited in the above articles and from the *E. coli* Genetic Stock Center at Yale University run by Dr. Barbara Bachman.

5.5.2.3. Construction Of Linear DNA Fragments Which Have Sequences Homologous To Closely Spaced Regions On The Chromosome In The Bacteria Which Flank Each Side Of The Gene Of Interest

The method whereby the DNA fragment is introduced into the chromosome to delete a gene or to regulate *recA* involves the construction of linear DNA fragments which have sequences homologous to closely spaced regions on the chromosome in the bacteria which flank each side of the gene of interest. In general, they must contain at least 80 to 100 nucleotides homologous to sequences flanking the gene to be deleted or the *recA* gene. An antibiotic resistant locus such as Kan^r, which encodes a protein making the bacterial resistant to the antibiotic, or other marker, is placed between these flanking sequences.

The linear DNA segment is introduced into a specific cell strain and selection is done for a double, reciprocal recombination which will delete the target gene and insert the marker into its place. As a result of the insertion of the antibiotic resistant gene, the cells are now resistant to the antibiotic. Cells which do not contain the insertion are eliminated by growing the bacteria in a medium containing the antibiotic.

Any other gene which allows for rapid screening of the cells containing a double, reciprocal recombination may be used in place of a gene for antibiotic resistance. For example, any gene for an essential protein, including enzymes, cofactors, proteins which are necessary for the synthesis of essential lipids, polysaccharides, nucleic acids, and other protein molecules such as receptors, as well as nucleic acids which have functional activity such as ribozymes, may be used. Other genes which confer a detectable phenotype on the cell strain such as sensitivity to temperature or ultraviolet radiation, auxotrophism for a sugar, amino acid, protein or nucleotide, or any other phenotype which can be detected by chemical indicators either in vitro or in vivo assay, or an immunoassay for a specific cellular component may also be used. Such chemical, radioactive, or immunological screening assays are well known to those skilled in the art.

5.5.3. Eliminating Contamination From The Host Producing Its Own Protein By Deletion And Replacement

In one application, in which the goal is to produce and purify a foreign protein, and the microorganism encodes its own version of the protein, the gene for the microorganism's own protein is eliminated. The gene for the foreign protein is then inserted and the protein produced. The purification process is thereby simplified since there is no contamination by the host protein, whether analogous to the protein being produced or unrelated which copurifies with, or interferes with the purification of, the protein being produced. For example, a protein may interfere with binding of the protein to be purified to a column.

5.5.4. A Plasmid Is Used To Introduce A Mutated Gene Into An Organism

In a second application, a plasmid is used to introduce a gene into an organism which typically contains a mutation in the gene to be investigated resulting in a negative phenotype for the product of the gene to be investigated. Failure of the organism to produce a biologically active form of the protein encoded by the mutated gene may confer a lethal phenotype under certain defined conditions. For example, this can be at a temperature, designated as the restrictive temperature, at which the mutant protein denatures or otherwise undergoes inactivation. The cloned gene which is introduced is selected for by virtue of its ability to confer cell viability or any other detectable phenotype for the desired protein at the restrictive temperature. The acquisition of viability

or other detectable phenotype at the normally restrictive temperature is evidence that the gene of interest has been cloned.

5.5.4.1. Problems: The Defective Host Protein May Interact With A Protein Produced From The Introduced Plasmid

The major problem with this second system is that, unless the inactive gene is deleted in entirety, the defective host protein may interact with a protein produced from the introduced plasmid. This interaction may stabilize the defective host protein enough so that its activity is restored even at the restrictive temperature. In this case, the restoration of growth at the restrictive temperature would be a false positive, that is, the growth would not be due to activity encoded by the cloned DNA segment. This problem holds true for all selections based on complementation of a phenotype which is due to a defect in a specific protein. Such phenotypes include temperature sensitivity, amino acid auxotrophies or any other auxotrophies which result from a lack of synthesis of a key ingredient such as a sugar, nucleotide, critical protein or nucleic acid, or cofactor used for oxidation, reduction, or transamination reactions.

5.5.5. False Positives

The problem of "false positives" also exists for cloned DNA pieces which are created to encode enzyme fragments as a means to define the catalytic core or to define any segment which achieves a specific purpose, such as a piece which undergoes self-association, binds to a specific ligand or receptor, or forms a specific complex or array with one or more additional components. In these cases, the engineering of protein fragments, which are tested in a host cell that encodes a defective version of the protein of interest, is seriously hampered if the defective host protein interacts in any way with the engineered pieces.

5.5.6. Use Of Temperature Sensitive Replicons

In the present invention, specifically designed linear DNA fragments are used to create a deletion of a gene by site-specific recombination. These fragments are transformed into the host cell. Cell viability or the detectable phenotype can be maintained during the procedure by provision of the gene encoding the desired protein on a recombinant plasmid that has a temperature-sensitive replicon, so that the cells which contain the deletion have a temperature sensitive phenotype. To achieve the deletion by recombination with the

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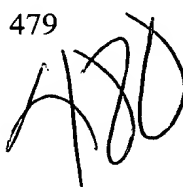
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sugarcane, tobacco, and arabidopsis; fish, algae, fungi (penicillium, aspergillus, podospora, neurospora, saccharomyces), insect (e.g., baculo lepidoptera), yeast (picchia and saccharomyces, Schizosaccharomyces pombe). Also of interest are many bacterial cell types, both gram- negative and gram-positive, such as Bacillus subtilis, B. licheniformis, B. cereus, Escherichia coli, Streptomyces, Pseudomonas, Salmonella, Actinomycetes, Lactobacillus, Acetobacter, Deinococcus, and Erwinia. The complete genome sequences of E. coli and Bacillus subtilis are described by Blattner et al., Science 277, 1454- 1462 (1997); Kunst et al., Nature 390, 249-256 (1997).

6.2. Screening for improved strains

Strains showing viability in initial selections are assayed more quantitatively for improvements in the desired properties before being restochastic &/or non-stochastically mutagenized with other strains.

Progeny resulting from mutagenesis of a strain, or those pre-selected for their ethanol tolerance and/or thermostability, can be plated on non-selective agar. Colonies can be picked robotically into microtiter dishes and grown. Cultures are replicated to fresh microtiter plates, and the replicates are incubated under the appropriate stress condition(s). The growth or metabolic activity of individual clones may be monitored and ranked. Indicators of viability can range from the size of growing colonies on solid media, density of growing cultures, or color change of a metabolic activity indicator added to liquid media. Strains that show the greatest viability are then mixed and stochastic &/or non-stochastically mutagenized, and the resulting progeny are rescreened under more stringent conditions



Abstract The purpose of this study was to determine the effect of a 12-week training program on the heart rate (HR) and blood pressure (BP) of sedentary, middle-aged men. The subjects were divided into two groups: a control group and an exercise group. The control group consisted of 10 men who did not exercise, and the exercise group consisted of 10 men who exercised for 12 weeks. The HR and BP were measured at baseline and at the end of the 12-week period. The results showed that the exercise group had a significant decrease in both HR and BP compared to the control group. The HR of the exercise group decreased from 72.5 ± 5.0 beats/min at baseline to 68.5 ± 4.0 beats/min at the end of the 12-week period. The BP of the exercise group decreased from 120/80 mmHg at baseline to 110/70 mmHg at the end of the 12-week period. The control group showed no significant change in HR or BP. These results suggest that a 12-week training program can effectively reduce HR and BP in sedentary, middle-aged men.

In some cases, the genes that encode rate-limiting steps in a biochemical process, or that contribute to a phenotype of interest are known. This method can be used to target family stochastic &/or non-stochastically mutagenized libraries to such loci, generating libraries of organisms with high quality family stochastic &/or non-stochastically mutagenized libraries of alleles at the locus of interest. An example of such a gene would be the evolution of a host chaperonin to more efficiently chaperone the folding of an overexpressed protein in *E. coli*.

Integration of multiple stochastic &/or non-stochastic mutagenized genes at multiple loci can be achieved using recursive cycles of integration (generating duplications), excision (leaving the improved allele in the chromosome) and transfer of additional evolved genes by serially applying the same procedure.

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Colonies with improved phenotype are screened (e.g., by mass spectroscopy for enzyme activity or small molecule production, or a chromogenic screen, or the like, depending on the phenotype to be assayed). Negative selection (i.e. sue selection) is imposed to force excision of tandem duplication. Roughly half C, of the colonies should retain the improved phenotype. Importantly, this process regenerates a “clean” chromosome in which the wild type locus is replaced with a family stochastic &/or non-stochastically mutagenized fragment that encodes a beneficial allele. Since the chromosome is "clean" (i.e., has no vector sequences), other improved alleles can also be moved into this point on the chromosome by homologous recombination.

Transductants are screened for further improvement in phenotype by virtue of receiving the transduced tandem duplication, which itself contains the family stochastic &/or non-stochastically mutagenized genetic material. Negative selection is again imposed and the process of stochastic &/or non-stochastically mutagenizing the improved strain is recursively repeated as desired.

1. *Chlorophyll a* (Chl *a*) content was determined using a spectrophotometer (Shimadzu UV-160U) at 663 nm. The concentration of Chl *a* was calculated using the following equation: $\text{Chl } a \text{ (mg/L)} = 12.7 \times \text{OD}_{663}$.

Screening can also be done by observing such aspects of growth as colony size, halo formation, etc. Additionally, screening for production of a desired compound, such as a therapeutic drug or "designer chemical" can be accomplished by observing binding of cell products to a receptor or ligand, such as on a solid support or on a column. Such screening can additionally be accomplished by binding to antibodies, as in an ELISA. In some instances the screening process is preferably automated so as to allow screening of suitable numbers of colonies or cells. Some examples of automated screening devices include fluorescence activated cell sorting, especially in conjunction with cells immobilized in agarose (see Powell et. al. *Bio/Technology* 8:333-337 (1990); Weaver et. al. *Methods* 2:234- 247 (1991)), automated ELISA assays, scintillation proximity assays (Hart, H.E. et al., *Molecular Immunol.* 16:265-267 (1979)) and the formation of fluorescent, coloured or UV absorbing compounds on agar plates or in microtitre wells (Krawiec, S., *Devel. Indust. Microbiology* 31:103-114 (1990)).

In particular, uncloned but differentially expressed proteins (e.g., those induced in response to new compounds, such as biodegradable pollutants in the medium) can be screened by differential display (Appleyard et al. *Mol. Gen. Genet.* 247:338-342 (1995)).

Hopwood (Phil Trans R. Soc. Lond B 324:549-562) provides a review of screens for antibiotic production. Omura (Microbio. Rev. 50:259-279 (1986) and Nisbet (Ann Rev. Med. Chem. 21:149-157 (1986)) disclose screens for antimicrobial agents, including supersensitive bacteria, detection of beta-lactamase and D,D- carboxypeptidase inhibition, beta-lactamase induction, chromogenic substrates and monoclonal antibody screens.

Antibiotic targets can also be used as screening targets in high throughput screening. Antifungals are typically screened by inhibition of fungal growth. Pharmacological agents can be identified as enzyme inhibitors using plates containing the enzyme and a chromogenic substrate, or by automated receptor assays. Hydrolytic enzymes (e.g., proteases, amylases) can be screened by including the substrate in an agar plate and scoring for a hydrolytic clear zone or by using a colorimetric indicator (Steele et al. Ann. Rev. Microbiol. 45:89-106 (1991)). This can be coupled with the use of stains to detect the effects of enzyme action (such as congo red to detect the extent of degradation of celluloses and hemicelluloses).

Tagged substrates can also be used. For example, lipases and esterases can be screened using different lengths of fatty acids linked to umbelliferyl. The action of lipases or esterases removes this tag from the fatty acid, resulting in a quenching or enhancement of umbelliferyl fluorescence. These enzymes can be screened in microtiter plates by a robotic device.

6.4.1. FACS

Fluorescence activated cell sorting (FACS) methods are also a powerful tool for selection/screening. In some instances a fluorescent molecule is made within a cell (e.g., green fluorescent protein). The cells producing the protein can simply be sorted by FACS. Gel microdrop technology allows screening of cells encapsulated in agarose microdrops (Weaver et al. Methods 2:234-247 (1991)). In this technique products secreted by the cell (such as antibodies or antigens) are immobilized with the cell that generated them. Sorting and collection of the drops containing the desired product thus also collects the cells that made the product, and provides a ready source for the cloning of the genes encoding the

3492 (1995)). These primers result in a large amount of random 3' ends, which can undergo homologous recombination when reintroduced into cells.

If the second round of recombination is to be performed *in vivo*, as is often the case, it can be performed in the cell surviving screening/selection, or the recombinant genes can be transferred to another cell type (e.g., a cell is type having a high frequency of mutation and/or recombination). In this situation, recombination can be effected by introducing additional DNA segment(s) into cells bearing the recombinant genes. In other methods, the cells can be induced to exchange genetic information with each other by, for example, electroporation. In some methods, the second round of recombination is performed by dividing a pool of cells surviving screening/selection in the first round into two subpopulations. DNA from one subpopulation is isolated and transfected into the other population, where the recombinant gene(s) from the two subpopulations recombine to form a further library of recombinant genes. In these methods, it is not necessary to isolate particular genes from the first subpopulation or to take steps to avoid random shearing of DNA during extraction. Rather, the whole genome of DNA sheared or otherwise cleaved into manageable sized fragments is transfected into the second subpopulation. This approach is particularly useful when several genes are being evolved simultaneously and/or the location and identity of such genes within chromosome are not known.

After the second round of recombination, recombinant genes conferring the desired phenotype are again selected. The selection process proceeds essentially as before. If a suicide vector bearing a selective marker was used in the first round of selection, the same vector can be used again. Again, a cell or pool of cells surviving selection is selected. If a pool of cells, the cells can be subject to further enrichment.

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6.5. Factors involved in discovery and development of new drugs

In the discovery and development of new drugs, it is a common strategy to first try to identify molecules or complexes of molecules, naturally occurring within cells, that are involved in producing symptoms of a disease. These naturally occurring molecules can be thought of as "targets." A second major part of the strategy is then to find molecules that bind to the targets. These molecules are candidates for drug development, on the theory that a molecule that binds to a target can modulate (inhibit or enhance) the function of the target, thereby causing a change in the biological status of the cell containing the target. The change caused in the cell (e.g., a change in phenotype towards wild type, or a change in growth rate) may be therapeutically beneficial to the animal or human host of the cell.

The genomics revolution, by determining the DNA sequences of great numbers of genes from many different organisms, has considerably broadened the possibilities for drug discovery by identifying, large numbers of molecules that are potential targets of drug action. These technical advances in genomics however, have posed an entirely new set of challenges. Specifically, how can one prove that a chosen target molecule is essential to maintaining the disease or disorder to be treated? That is, how does one validate a target? Although methods currently available to validate targets do provide some guidelines in selection of drug targets, they are usually not conducted under the conditions in which a drug actually interacts with its target, and therefore provide a limited set of information. In addition, they do not directly address, among other things: 1) if a wild type (normal) target is essential for cell growth and viability during the disease state; 2) if the wild type gene products themselves are suitable targets for drug discovery; 3) if specific sites on a target are suitable for drug interaction (for example, in a pathogenic organism, there can be one gene coding for a single protein target with two activities -- one activity essential for growth and infectivity, the second activity non-essential); 4) if a compensatory mechanism in the cell, either in vitro or in vivo, can overcome or compensate for target modulation or, 5) if a disease state can be cured by modulation of function of the candidate target. These methods also do not provide a direct route for testing wild type target proteins in high throughput screening assays.

6.6. General approach for identifying targets

to produce, upon expression of the gene encoding the biomolecular binder, a phenotypic effect in the cells (e.g., inhibition of growth), wherein the test of the constructed cells can be a test of the cells in culture or a test of the cells after introducing them into host animals, or both, and further, identifying, for a biomolecular binder that caused the phenotypic effect, one or more compounds that compete with the biomolecular binder for binding to the target cell component.

A test of the constructed cells after introducing them into host animals is especially well-suited to assessing whether a biomolecular binder can produce a particular phenotype by the expression (regulatable by the researcher) of a gene encoding the biomolecular binder. In this method, cells are constructed which have a gene encoding the biomolecular binder, and wherein the biomolecular binder can be produced by regulation of expression of the gene. The constructed cells are introduced into a set of animals. Expression of the gene encoding the biomolecular binder is regulated in one group of the animals (test animals) such that the biomolecular binder is produced. In another group of animals, the gene encoding the biomolecular binder is regulated such that the biomolecular binder is not produced (control animals). The cells in the two groups of animals are monitored for a phenotypic change (for example, a change in growth rate). If the phenotypic change is observed in cells in the test animals and not in the cells in the control animals, or to a lesser extent in the control animals, then the biomolecular binder has been proven to be effective in binding to its target cell component under in vivo conditions.

A further embodiment of the invention is a method for determining whether a target cell component of a particular cell type (a "first cell") is essential to producing a phenotypic effect on the first cell, the method having the steps: isolating the target component of the first cell; identifying a biomolecular binder of the isolated target component of the first cell; constructing a second type of cells ("second cell") comprising the target component and a regulable, exogenous gene encoding the biomolecular binder; and testing the second cell in culture for an altered phenotypic effect, upon production of the biomolecular binder in the second cell; whereby, if the second cell shows the altered phenotypic effect upon production of the biomolecular binder, then the target component of the first cell is essential to producing the phenotypic effect on the first cell. The target cell component in this embodiment and in other embodiments not limited

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control cells, then the biomolecule is a biomolecular inhibitor of growth of the pathogen cells.

6.6.1.1.2. Identifying compounds that inhibit infection of a mammal by a pathogen

A further embodiment of the invention is a method, employing an animal test, for identifying one or more compounds that inhibit infection of a mammal by a pathogen by binding to a target cell component, comprising constructing a pathogen comprising a regulable gene encoding a biomolecule which binds to the target cell component, infecting test animals with the pathogen, regulating expression of the regulable gene to produce the biomolecule, monitoring the test animals and suitable control animals for signs of infection, wherein observing fewer or less severe signs of infection in the test animals than in suitable control animals indicates that the biomolecule is a biomolecular inhibitor of infection, and identifying one or more compounds that compete with the biomolecular inhibitor of growth for binding to the target cell component (as by employing a competitive binding assay), then the compound inhibits infection of a mammal by a pathogen by binding to a target.

The competitive binding assay to identify binding analogs of biomolecular binders, which have been proven to bind to their targets in an intracellular test of binding, can be applied to any target for which a biomolecular binder has been identified, including targets whose function is unknown or targets for which other types of assays are not easily developed and performed. Therefore, the method of the invention offers the advantage of decreasing assay development time when using a gene product of known function as a target cell component and the advantage of bypassing the major hurdle of gene function identification when using a gene product of unknown function as a target cell component.

Other embodiments of the invention are cells comprising a biomolecule and a target cell component, wherein the biomolecule is produced by expression of a regulable gene, and wherein the biomolecule modulates function of the target cell component, thereby causing a phenotypic change in the cells. Yet other embodiments are cells comprising a biomolecule and a target cell component, wherein the biomolecule is a biomolecular binder of the target cell component, and is encoded by a regulable gene. The cells can

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A phenotypic effect can be the causing or curing of a disease state, especially where mammalian cells are referred to herein. For cells of a pathogen or tumor cells, especially, a phenotypic effect can be the slowing of growth rate or cessation of growth.

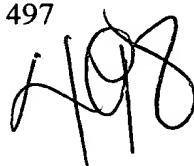
Biomolecule: a molecule which can be produced as a gene product in cells that have been appropriately constructed to comprise one or more genes encoding the biomolecule. Preferably, production of the biomolecule can be turned on, when desired, by an inducible promoter. A biomolecule can be a peptide, polypeptide, or an RNA or DNA oligonucleotide, but is preferably a peptide. The same biomolecules can also be made synthetically. For peptides, see Merrifield, J., J. Am. Chem. Soc. 85: 2140-2154 (1963). For instance, an Applied Biosystems 431 A Peptide Synthesizer (Perkin Elmer) can be used for peptide synthesis. Biomolecules produced as gene products intracellularly are tested for their interaction with a target in the intracellular steps described herein (tests performed with cells in culture and tests performed with cells that have been introduced into animals). The same biomolecules produced synthetically are tested for their binding to an isolated target in an initial in vitro method described herein.

Synthetically produced biomolecules can also be used for a final step of the method for finding compounds that are competitive binders of the target.

Biomolecular Binder (of a target): a biomolecule which has been tested for its ability to bind to an isolated target cell component in vitro and has been found to bind to the target.

Biomolecular Inhibitor of Growth: a biomolecule which has been tested for its ability to inhibit the growth of cells constructed to produce the biomolecule in an "in culture" test of the effect of the biomolecule on growth of the cells, and has been found, in fact, to inhibit the growth of the cells in this test in culture.

Biomolecular Inhibitor of Infection: a biomolecule which has been tested for its ability to ameliorate the effects of infection, and has been found to do so. In the test, pathogen cells constructed to regulably express the biomolecule are introduced into one or more animals, the gene encoding the biomolecule is regulated so as to allow production of the



[illegible]

In many circumstances the isolated material will form part of a composition (for example, a more or less crude extract containing other substances), buffer system or reagent mix. In other circumstances, the material may be purified to essential homogeneity, for example as determined by PAGE or column chromatography (for example, HPLC).

Pathogen or Pathogenic Organism: an organism which is capable of causing disease, detectable by signs of infection or symptoms characteristic of disease. Pathogens can include procaryotes (which include, for example, medically significant Gram- positive bacteria such as *Streptococcus pneumoniae*, *Enterococcus faecalis* and *Staphylococcus aureus*, Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, and "acid-fast" bacteria such as *Mycobacteria*, especially *M. tuberculosis*), eucaryotes such as yeast and fungi (for example, *Candida albicans* and *Aspergillus fumigatus*) and parasites. It should be recognized that pathogens can include such organisms as soil-dwelling organisms and "normal flora" of the skin, gut and orifices, if such organisms colonize and cause symptoms of infection in a human or other mammal, by abnormal proliferation or by growth at a site from which the organism cannot usually be cultured.

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(which can be regulable) of a gene in the cell. As used in general step (2), a biomolecule is a gene product (e.g., polypeptide, RNA, peptide or RNA oligonucleotide) of an exogenous gene -- a gene which has been introduced in the course of construction of the cell.

Biomolecules that bind to and alter the function of a candidate target are identified by various in vitro methods. Upon production of the biomolecule within a cell either in vitro or within an animal model system, the biomolecule binds to a specific site on the target, alters its intracellular function, and hence produces a phenotypic change (e.g. cessation of growth, cell death). When the biomolecule is produced in engineered pathogen cells in an animal model of infection, cessation of growth or death of the engineered pathogen cells leads to the clearing of infection and animal survival, demonstrating the importance of the target in infection and thereby validating the target.

6.7.1.1. Comprising an exogenous regulable gene encoding the biomolecule

A method for (1) identifying a biomolecule that produces a phenotypic effect on a cell (wherein the cell can be, for instance, a pathogen cell or a mammalian cell) and (2) simultaneous intracellular target validation, can comprise steps of introducing into an animal a cell comprising an exogenous regulable gene encoding the biomolecule, regulating expression of the gene to produce the biomolecule in the cell, and monitoring said cell in the animal for a phenotypic effect, compared to a suitable control cell. If the cell of this test manifests a phenotypic effect, this indicates that the biomolecule produced in the cell causes a phenotypic effect on the cell. If this phenotypic effect is the inhibition of growth of the cells, then the biomolecule can be termed a "biomolecular inhibitor" or a "biomolecular inhibitor of growth." It may be desirable to perform another test of intracellular function, using cell culturing techniques, wherein the cell comprising an exogenous regulable gene encoding the biomolecule of interest, and comprising the target cell component, is treated so as to turn on expression of the gene encoding the biomolecule, and one or more phenotypic characteristics of the cells in culture are monitored relative to suitable control cells, where the control cells do not produce the biomolecule. It may be preferable, where both "in culture" and "in animal" intracellular tests are performed, to do an "in culture" test first.

6.7.1.2. Advantages of intracellular validation

The purpose of intracellular validation for the combination of a potential target for drug action and molecule for drug development is two-fold. First, it demonstrates that the biomolecule under study produces a phenotypic effect on a living cell. In contrast with conditions in an in vitro binding test, the biomolecule in an intracellular test is exposed to a multitude of potential binding partners in the living cell, and interaction with one or more of these binding partners in the cell may be unproductive or result in undesirable effects. These effects are not detectable in an in vitro binding test. Second, where a biomolecule has been shown previously by in vitro tests to bind to a target cell component (that is, the biomolecule can be called a "biomolecular binder" of the target cell component, intracellular validation provides proof that the target cell component is essential to the maintenance of the original phenotype of the cell. Therefore, the target is validated for drug discovery and the biomolecule can then be utilized in a competitive binding assay to identify compounds that will have an effect on target molecule function.

Efficient binding between a biomolecule and a target cell component may be demonstrated in vitro; even binding, that inhibits activity of a target enzyme may be demonstrated in vitro. However, in the living cells, there could exist a redundant system that nullifies the effect of the biomolecule binding to the target cell component. For example, production of an enzyme having similar activity to that of the target cell component may be induced in the cells. By a mechanism such as this, the cell could escape any effect the biomolecule might otherwise cause by binding to the target cell component.

6.7.1.2.1. Ensures that the target cell component is in its natural conformation as seen in the disease state

Using an intracellular test to validate biomolecule/target cell component interaction is superior to using only an in vitro test using isolated molecules, because the intracellular test ensures that the target cell component is in its natural conformation and that the biomolecule "sees" the target cell component in that conformation, as that conformation occurs in a disease state. That in an intracellular test a biomolecule finds a site which ultimately causes a phenotypic effect on the cell indicates that the biomolecule is binding



to a functionally relevant site on the target cell component (e.g., an active site of an enzyme). Thus, molecules that are found to be structural analogs of the biomolecule and to compete with the biomolecule for a binding site on the target will also interact with the functionally relevant site of the target cell component, as functional analogs. A functional analog of the biomolecule can be found through competitive binding assays of the biomolecule against compounds (as in a library of compounds) that are potential binders of the target cell component. Structural analogs can also be found by rational drug design once a biomolecular binder is identified, by designing drugs that mimic the structure of the biomolecular binder. These structural analogs can be tested for their binding properties by techniques described herein.

6.7.1.2.2. Biomolecule does not have to pass through a cell membrane or rely on inefficient uptake mechanisms of the cell

In further steps following one or more intracellular tests of the biomolecule/target cell component combination, one or more compounds that can also produce the phenotypic effect caused by the biomolecule can be identified in an in vitro competitive binding assay (which may be adapted for high- throughput screening) as compounds that compete with the biomolecule for a binding site on the target cell component. Target cell components can be isolated from the type of cell in which the phenotypic effect is desired (for instance, cells of pathogenic bacteria, yeast or fungi; mammalian cells, such as tumor cells), or from cells engineered to produce the cell component or a derivative of the cell component that would provide (at least some) structurally identical binding sites (e.g., a fusion protein). Compounds that produce the phenotypic effect observed with the biomolecule can be found in the competitive binding assay upon screening of libraries of compounds (for example, small molecule compounds or natural products or libraries that can be selected for having as their members compounds that have greater intracellular stability than biomolecules such as peptides or RNA oligonucleotides).

6.7.1.3. Methods for identifying compounds that inhibit the growth of cells having a target cell component

Described herein are similar methods for identifying inhibitors of target molecules or target cell components of pathogenic organisms. These methods can include a target validation procedure using an animal model for confirming that a cell component of a

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pathogenic organism is essential, after infection with the organism has been established in a host, and that the inhibitor is effective against the organism after the organism has established the infection. A goal of the procedure is to identify compounds and/or gain the knowledge required to design compounds that can be used as antimicrobial agents to treat a human or other mammal having an infection of the organism.

6.7.1.4. Methods for in vitro and in vivo validation of target and assay combinations

The invention provides methods for in vitro and in vivo validation of target and assay combinations. Following selection of biomolecular binders to the isolated target cell component of interest, the invention can incorporate steps for (1) regulable (e.g., inducible) intracellular expression of a gene encoding the biomolecular binder and (2) monitoring cell viability in culture (e.g., cell growth in liquid media or agar plates) or in vivo (e.g., growth of introduced cells or pathogen virulence in an animal infection model) or both. If intracellular expression of the biomolecular binder inhibits the function of a target essential for growth (presumably by binding to the target at a biologically relevant site) cells monitored in step (2) will exhibit a slow growth or no growth phenotype. Targets found to be essential for growth by these methods are validated starting points for drug discovery, and can be incorporated into assays to identify more stable compounds that bind to the same site on the target as the biomolecule. Where the cells are pathogen cells and the desired phenotypic change to be monitored is inhibition of growth, the invention provides a procedure to examine the activity of target (pathogen) cell components in an animal infection model.

6.7.1.5. Mimicking the environment for traditional antimicrobial therapy

Controlled expression in cells of biomolecular binders to the target of interest mimics the environment for traditional antimicrobial therapy and validates targets as essential and appropriate for drug discovery. The technology facilitates choosing the best antimicrobial targets for drug discovery by facilitating, direct observation of the effect (phenotype) produced by target inhibition at a specific target subsite. The process is broadly applicable to a variety of targets. The process also validates target and biomolecular binder combinations as a direct path to high throughput screening for binding analogs of the

biomolecular binder, and is equally facile with targets that are gene products of genes of unknown function or genes of known function. Validated target and biomolecular binder assay combinations can be used directly in in vitro or in vivo competitive binding assays for screening chemical compound files. Compounds that compete with the biomolecular binders are identified as potential medicinal chemistry leads.

6.7.1.6. Study as a target cell component a gene product of a particular cell type

6.7.1.7. Target cell component can be produced recombinantly

6.7.1.8. Method should be used with target cell components which have not been previously isolated or characterized and whose functions are unknown

It is an advantage of the target validation method that it can be used with target cell components which have not been previously isolated or characterized and whose functions are unknown. In this case, a segment of DNA containing an open reading frame (ORF; a cDNA can also be used, as appropriate to a eukaryotic cell) which has been isolated from a cell of a type that is to be an object of drug action (e.g., tumor cell, pathogen cell) can be cloned into a vector, and the target gene product of the ORF can be produced in host cells harboring the vector. The gene product can be purified and further studied in a manner similar to that of a gene product that has been previously isolated and characterized.

6.7.1.9. Host cells

component so produced can then be isolated from the host cells. Many protein purification methods are known that separate proteins on the basis of, for instance, size, charge, or affinity for a binding partner (e.g., for an enzyme, a binding partner can be a substrate or substrate analog), and these methods can be combined in a sequence of steps by persons of skill in the art to produce an effective purification scheme. For methods to manipulate RNA, see, for example, Chapter 4 in Current Protocols in Molecular Biology (Ausubel, F.M. et al., eds), John Wiley & Sons, New York, 1998.

An isolated cell component or a fusion protein comprising the cell component can be used in a test to identify one or more biomolecular binders of the isolated product (general step (1)). A biomolecular binder of a target cell component can be identified by in vitro assays that test for the formation of complexes of target and biomolecular binder noncovalently, bound to each other. For example, the isolated target can be contacted with one or more types of biomolecules under conditions conducive to binding, the unbound biomolecules can be removed from the targets, and a means of detecting bound complexes of biomolecules and targets can be applied. The detection of the bound complexes can be facilitated by having either the potential biomolecular binders or the target labeled or tagged with an adduct that allows detection or separation (e. g., radioactive isotope or fluorescent label; streptavidin, avidin or biotin affinity label).

Alternatively, both the potential biomolecular binders and the target can be differentially labeled. For examples of such methods see, e.g., WO 98/19162.

6.7.1.10. Biomolecules to be tested and means for detection

The biomolecules to be tested for binding to a target can be from a library of candidate biomolecular binders, (e.g., a peptide or oligonucleotide library). For example, a peptide library can be displayed on the coat protein of a phage (see, for examples of the use of genetic packages such as phage display libraries, Koivunen, E. et al., J Biol. Chem. 268:20205-20210 (1993)). The biomolecules can be detected by means of a chemical tag or label attached to or integrated into the biomolecules before they are screened for binding properties. For example, the label can be a radioisotope, a biotin tag, or a

specifically bound biomolecules. In this aspect, elution buffer can be formulated to permit retention of the fusion protein by the affinity matrix, but can be formulated to interfere with binding of the test biomolecule(s) to the target portion of the fusion protein. For example, a change in the ionic strength or pH of the elution buffer can lead to release of biomolecules, or the elution buffer can comprise a release component or components designed to disrupt binding of biomolecules to the target portion of the fusion protein.

Immobilization can be performed prior to, simultaneous with, or after contacting, the fusion protein with biomolecule, as appropriate. Various permutations of the method are possible, depending upon factors such as the biomolecules tested, the affinity matrix-ligand pair selected, and elution buffer formulation. For example, after the wash step, fusion protein with biomolecules bound thereto can be eluted from the affinity matrix with a suitable elution buffer (a matrix elution buffer, such as glutathione for a GST fusion). Where the fusion protein comprises a cleavable linker, such as a thrombin cleavage site, cleavage from the affinity ligand can release a portion of the fusion with the biomolecules bound thereto. Bound biomolecule can then be released from the fusion protein or its cleavage product by an appropriate method, such as extraction.

6.7.1.12. Various methods to identify biomolecular binders

One or more candidate biomolecular binders can be tested simultaneously. Where a mixture of biomolecules is tested, the biomolecules selected by the foregoing processes can be separated (as appropriate) and identified by suitable methods (e.g., PCR, sequencing, chromatography). Large libraries of biomolecules (e.g., peptides, RNA oligonucleotides) produced by combinatorial chemical synthesis or other methods can be tested (see e. a., Ohlmeyer, M.H.J. et al., Proc. Natl. Acad. Sci. USA 90:10922-10926 (1993) and DeWitt, S.H. et al., Proc. Natl. Acad. Sci. USA 90:6909-6913 (1993), relating to tagged compounds; see also Rutter, W.J. et al. U.S. Patent No. 5,010,175; Huebner, V.D. et al., U.S. Patent No. 5,182,366; and Geysen, H.M., U.S. Patent No. 4,833,092). Random sequence RNA libraries (see Ellington, A.D. et al., Nature 346:818-822 (1990); Bock, L.C. et al., Nature 355:584-566 (1992); and Szostak, J.W., Trends in Biochem. Sci. 17:89-93 (March, 1992)) can also be screened according to the present method to select

6.7.1.13. Host cells: Engineered to control expression

Host cells (also, "second cells" in the terminology used above) of the cell type (e.g., species of pathogenic bacteria) the target was isolated from (or the gene encoding the target was originally isolated from, if the target is produced by recombinant methods), can be engineered to harbor a gene that can regulably express the biomolecular binder (e.g., under an inducible or repressible promoter). The ability to regulate the expression of the biomolecular binder is desirable because constitutive expression of the biomolecular binder could be lethal to the cell.

Therefore, inducible or regulated expression gives the researcher the ability to control if and when the biomolecular binder is expressed. The gene expressing the biomolecular binder can be present in one or more copies, either on an extra chromosomal structure, such as on a single or multicopy plasmid, or integrated into the host cell genome. Plasmids that provide an inducible gene expression system in pathogenic organisms can be used. For example, plasmids allowing tetracycline-inducible expression of a gene in *Staphylococcus aureus* have been developed.

6.7.1.14. Genes for expression

For intracellular expression of a biomolecule to be tested for its phenotypic effect in a eukaryotic cell (e.g., mammalian cell), the genes for expression can be carried on plasmid-based or virus-based vectors, or on a linear piece of DNA or RNA. For examples of expression vectors, see Hosfield and Lu, *Biotechniques*: 306-309, 1998; Stephens and Cockett, *Nucleic Acid Research* 17:7110, 1989; Wohlgemuth et al, *Gene Therapy*, 3:503-512, 1996; Ramirez-Solis et al, *Gene* 87:291-294, 1990, Dirks et al, *Gene* 149:387-388, 1994; Chenaalvala et al. *Current Opinion in Biotechnologies* 2:718-722, 1991; *Methods in Enzymology*, Volume 185, (D.V. Goeddel, ed.) Academic Press, San Diego, 1990. The genetic material can be introduced into cells using a variety of techniques, including whole cell or protoplast transformation, electroporation, calcium phosphate-DNA precipitation or DEAE- Dextran transfection, liposome mediated DNA or RNA transfer, or transduction with recombinant viral or retroviral vectors. Expression of the gene can be constitutive (e.g., ADHI promoter for expression in *S. cerevisiae* (Bennetzen, J.L. and Hall, B.D., J

Biol. Chem 257:3026-3031 (1982)), or CMV immediate early promoter and RSV LTR for mammalian expression) or inducible, as the inducible GAL I promoter in yeast (Davis, L.I. and Fink, G.R., Cell 61:965-978 (1990)). A variety of inducible systems can be utilized, for example, E. coli Lac repressor/operator system and Tn10 Tet repressor/operator systems have been engineered to govern regulated expression in organisms from bacterial to mammalian cells. Regulated gene expression can also be achieved by activation. For example, gene expression governed by HIV LTR can be activated by HIV or SIV Tat proteins in human cells; GAL4 promoter can be activated by galactose in a nonglucose-containing medium. The location of the biomolecule binder genes can be extra chromosomal or chromosomally integrated. The chromosome integration can be mediated through homologous or nonhomologous recombinations.

For proper localization in the cells, it maybe desirable to tag the biomolecule binders with certain peptide signal sequences (for example, nuclear localization signal (NLS) sequences, mitochondria localization sequences). Secretion sequences have been well documented in the art.

6.7.1.15. Fused biomolecular binders

For presentation of the biomolecular binders in the intracellular system, they can be fused N-terminally, C-terminally, or internally in a carrier protein (if the biomolecular binder is a peptide), and can be fused (5', 3' or internally) in a carrier RNA or DNA molecule (if the biomolecular binder is a nucleic acid). The biomolecular binder can be presented with a protein or nucleic acid structural scaffold. Certain linkages (e.g., a 4-glycine linker for a peptide or a stretch of A's for an RNA can be inserted between the biomolecular binder and the carrier proteins or nucleic acids.

In such engineered cells, the effect of this biomolecular binder on the phenotype of the cells can be tested, as a manifestation of the binding (implying binding to a functionally relevant site, thus, an activator, or more likely, an inhibitory) effect of the biomolecular binder on the target used in an in vitro binding assay as described above. An intracellular test can not only determine which biomolecular binders have a phenotypic effect on the cells, but at the same time can assess whether the target in the cells is essential for

6.7.1.16. Engineering strain of cells

A further test can, again, employ an engineered strain of cells that comprise both the target cell component and one or more genes encoding a biomolecule tested to be a biomolecular binder of the target cell component. The cells of the cell strain can be tested in animals to see if regulable expression of the biomolecular binder in the engineered cells produces an observable or testable change in phenotype of the cells. Both the "in culture" test for the effect of intracellular expression of the biomolecular binder and the "in animal" test (described below) for the effect of intracellular expression of the biomolecular binder can be applied not only towards drug discovery in the categories of antimicrobials and anticancer agents, but also towards the discovery of therapeutic agents to treat inflammatory diseases, cardiovascular diseases, diseases associated with metabolic pathways, and diseases associated with the central nervous system, for example.

Where the engineered strain of cells is a strain of pathogen cells or tumor cells, the object of the test is to see whether production of the biomolecular binder in the engineered strain inhibits growth of these cells after their introduction into an animal by the engineered pathogen. Such a test can not only determine which biomolecular binders are inhibitors of growth of the cells, but at the same time can assess whether the target in the cells is essential for maintaining growth of the cells (infection, for a pathogenic organism) in a host mammal. Suitable animals for such an experiment are, for example, mammals such as mice, rats, rabbits, guinea pigs, dogs, pigs, and the like. Small mammals are preferred for reasons of convenience.

The engineered cells are introduced into one or more animals ("test" animals) and into one or more animals in a separate group ("control" animals) by a route appropriate to cause symptoms of systemic or local growth of the engineered cells.

The route of introduction may be, for example, by oral feeding, by inhalation, by subdermal, intramuscular, intravenous, or intraperitoneal injection as appropriate to the desired result.

compounds can take advantage of known methods to identify competing molecules in a binding assay. These steps comprise general step (3) of the method.

In one method to identify such compounds, a biomolecular inhibitor (or activator) can be contacted with the isolated target-cell component to allow binding, one or more compounds can be added to the milieu comprising the biomolecular inhibitor and the cell component under conditions that allow interaction and binding between the cell component and the biomolecular inhibitor, and any biomolecular inhibitor that is released from the cell component can be detected.

6.7.2.1. Fluorescence

One suitable system that allows the detection of released biomolecular inhibitor (or activator) is one in which fluorescence polarization of molecules in the milieu can be measured. The biomolecular inhibitor can have bound to it a fluorescent tag or label such as fluorescein or fluorescein attached to a linker.

Assays for inhibition of the binding of the biomolecular inhibitor to the cell component can be done in microtiter plates to conveniently test a set of compounds at the same time. In such assays, a majority of the fluorescently labeled biomolecular inhibitor must bind to the protein in the absence of competitor compound to allow for the detection of small changes in the bound versus free probe population when a compound which is a competitor with a biomolecular inhibitor is added (B.A. Lynch, et al., Analytical Biochemistry 247:77-82 (1997)). If a compound competes with the biomolecular inhibitor for a binding site on the target cell component, then fluorescently labeled biomolecular inhibitor is released from the target cell component, lowering the polarization measured in the milieu.

6.7.2.2. Radioactive isotope

In a further method for identifying one or more compounds that compete with a biomolecular inhibitor (or activator) for a binding site on a target cell component, the target cell component can be attached to a solid support, contacted with one or more

compounds, and contacted with the biomolecular inhibitor. One or more washing steps can be employed to remove biomolecular inhibitor and compound not bound to the cell component. Either the biomolecular inhibitor bound to the target cell component or the compound bound to the target cell component can be measured. Detection of biomolecular inhibitor or compound bound to the cell compound can be facilitated by the use of a label on either molecule type, wherein the label can be, for instance, a radioactive isotope either incorporated into the molecule itself or attached as an adduct, streptavidin or biotin, a fluorescent label or a substrate for an enzyme that can produce from the substrate a colored or fluorescent product. An appropriate means of detection of the labeled biomolecular inhibitor or compound moiety of the biomolecular inhibitor- cell component complex or the compound-cell component complex can be applied. For example, a scintillation counter can be used to measure radioactivity. Radio labeled streptavidin or biotin can be allowed to bind to biotin or streptavidin, respectively, and the resulting complexes detected in a scintillation counter. Alkaline phosphatase conjugated to streptavidin can be added to a biotin-labeled biomolecular inhibitor or compound. Detection and quantitation of a biotin-labeled complex can then be by addition of pNPP substrate of alkaline phosphatase and detection by spectrophotometry, of a product which absorbs UV light at a wavelength of 405 nm. A fluorescent label can also be used, in which case detection of fluorescent complexes can be by a fluorometer. Models are available that can read multiple samples, as in a microtiter plate.

For example, in one type of assay, the method for identifying compounds comprises attaching the target cell component to a solid support, contacting the biomolecular inhibitor with the target cell component under conditions suitable for binding of the biomolecular inhibitor to the cell component, removing unbound biomolecular inhibitor from the solid support, contacting one or more compounds (e.g., a mixture of compounds) with the cell component under conditions suitable for binding of the biomolecular inhibitor to the cell component, and testing for unbound biomolecular inhibitor released from the cell component, whereby if unbound biomolecular inhibitor is detected, one or more compounds that displace or compete with the biomolecular inhibitor for a particular site on the target cell component have been identified.

Other methods for identifying compounds that are competitive binders with the biomolecule for a target can employ adaptations of fluorescence polarization methods. See, for instance, Anal. Biochem. 253(2):210-218 (1997), Anal. Biochem. 249(1):29-36 (1997), BioTechniques 17(3):585-589 (1994) and Nature 373:254-256 (1995).

Those compounds that bind competitively to the target cell component can be considered to be drug candidates. Further appropriate testing can confirm that those compounds which bind competitively with biomolecular inhibitors (or activators) possess the same activity as seen in an intracellular test of the effect of the biomolecular inhibitor or activator upon the phenotype of cells. Derivatives of these compounds having modifications to confer improved solubility, stability, etc., can also be tested for a desired phenotypic effect.

6.7.3. Combining steps

Combining steps for testing the phenotypic effects of a biomolecule, as can be produced in an intracellular test, with steps for identifying compounds that compete with the biomolecule for sites on a target cell component, yields a method for identifying a compound which is a functional analog of a biomolecule which produces a phenotypic effect on a cell. These steps can be to test, for the phenotypic effect, either in culture or in an animal model, or in both, a cell which produces a biomolecule by regulable expression of an exogenous gene in the cell, and to identify, if the biomolecule caused the phenotypic effect, one or more compounds that compete with the biomolecule for binding to a target cell component. If a compound is found to compete with the biomolecule for binding to the target cell component, then the compound is a functional analog of a biomolecule which produces a phenotypic effect on the cell. Such a functional analog can cause qualitatively a similar effect on the cell, but to a similar degree, lesser degree or greater degree than the biomolecule.

6.7.3.1. Method for determining whether a target component of a cell is essential to producing a phenotypic effect on the cell

A further embodiment of the invention combining general steps (1) and (2) is a method for determining whether a target component of a cell is essential to producing a phenotypic

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subcutaneously (s.c.) with the minimal tumorigenic dose for 3T3T-VITA or 3T3T-VITA control cells to develop fibroblastoma. One week after injection, group 1 mice start receiving MTX s.c. at 2 mg/kg/day as positive control, group 2 to 5 start receiving 1, 2, 5, or 10 mg/kg/day of tetracycline, group 6 start receiving saline (vehicle) as control. Five weeks after the introduction of cells, all of the mice are sacrificed and tumors are removed from them. Tumor mass is measured and compared among the groups.

An effective peptide identified by these in vivo experiments can be used for screening libraries of compounds to identify those compounds that competitively bind to DHFR. One mechanism of tumorigenesis is overexpression of proto-oncogenes such as Ha-ras (Reviewed by Suarez, H.G., *Anticancer Research* 9(5):1331-1343 (1989)).

Compounds that inhibit the activities of the products of such proto- oncogenes can be used for cancer chemotherapy. What follows is a further illustration of the methods described herein, as applied to mammalian cells.

Transgenic mice that overexpress human Ha-ras have been produced. Such transgenic mice develop salivary and/or mammary adenocarcinomas (Nielsen, L.L. et al, *In Vivo* 8(5):1331-1343 (1994)). Secondary transgenic mice that express rtTA can be generated using the pTet-On plasmid from Clontech.

Human Ha-ras open reading frame cDNA (Genbank Accession #GO0277) is amplified by RT-PCR using polyA- RNA isolated from human mammary gland or other tissues. Active Ha-ras is expressed using the BacPAK Baculovirus Expression System (Clontech) or other appropriate systems. The expressed Ha-ras is purified and biotinylated and subjected to peptide binder identification as exemplified herein for bacterial proteins as target cell components. The identified peptides are biochemically characterized for in vitro inhibition of Ha-ras GTPase activity.

Peptides that inhibit Ha-ras are cloned into plasmid pTPE (Clontech) for regulated expression as an N-terminal fusion of GST. Such constructs are used to generate tertiary transgenic mice using the secondary transgenic mice. Transgenic mice that are able to

overexpress peptide genes are identified by Northern and Western analysis. Control mice that express GST are also identified.

Various doses of tetracycline are administered to the tertiary transgenic mice by s.c. or i.p. injection before or after tumor onset. Prevention or regression of tumors resulting from expression of the peptide genes are analyzed as described above for murine fibroblastoma. Peptides found to be effective in in vivo experiments will be used to screen compounds that inhibit human Ha-ras activity for cancer therapy.

6.7.4. Disease targets

The method of the invention can be applied more generally to mammalian diseases caused by: (1) loss or gain of protein function, (2) over- expression or loss of regulation of protein activity. In each case the starting point is the identification of a putative protein target or metabolic pathway involved in the disease. The protocol can sometimes vary with the disease indication, depending on the availability of cell culture and animal model systems to study the disease. In all cases the process can deliver a validated target and assay combination to support the initiation of drug discovery.

Appropriate disease indications include, but are not limited to, Alzheimer's, arthritis, cancer, cardiovascular diseases, central nervous system disorders, diabetes, depression, hypertension, inflammation, obesity and pain.

Appropriate protein targets putatively linked to disease indications include, but are not limited to (1) the leptin protein, putatively linked to obesity and diabetes; (2) a mitogen-activated protein kinase putatively linked to arthritis, osteoporosis and atherosclerosis; (3) the interleukin-1 beta converting protein putatively linked to arthritis, asthma and inflammation; (4) the caspase proteins putatively linked to neurodegenerative diseases such as Alzheimer's, Parkinson's and stroke, and (5) the tumor necrosis factor protein putatively linked to obesity and diabetes. Appropriate protein targets include also, but are not limited to, enzymes catalyzing the following types of reactions: (1) oxido-reductases, (2) transferases, (3) hydrolases, (4) lyases, (5) isomerases, and (6) ligases.

The arachidonic acid pathway constitutes one of the main mechanisms for the production of pain and inflammation. The pathway produces different classes of end products, including the prostaglandins, thromboxane and leukotrienes.

Prostaglandins, an end product of cyclooxygenase metabolism, modulate immune function, mediate vascular phases of inflammation and are potent vasodilators. The major therapeutic action of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) is proposed to be inhibition of the enzyme cyclooxygenase (COX). Anti-inflammatory potencies of different NSAIDs have been shown to be proportional to their action as COX inhibitors. It has also been shown that COX inhibition produces toxic side effects such as erosive gastritis and renal toxicity. The knowledge base regarding the toxic side effects of COX inhibitors has been gained through years of monitoring human therapies and human suffering. Two kinds of COX enzymes are now known to exist, with inhibition of COX 1 related to toxicity, and inhibition of COX2 related to reduction of inflammation. Thus, selective COX2 inhibition is a desirable characteristic of new anti-inflammatory drugs. The method of the invention can provide a route from identification of potential drug targets to validating these targets (for example, COX1 and COX2) as playing a role in disease (pain and inflammation) to an examination of the phenotype for the inhibition of one or both target isozymes without human suffering. Importantly, this information can be collected in vivo.

As an alternative strategy, the method of the invention can be used to define the phenotype of "genes of unknown function" obtained from various human genome sequencing projects or to assess the phenotype resulting, from inhibition of one isozyme subtype or one member of a family of related protein targets.

6.8. Biological Chips

6.8.1. General Considerations

In one aspect the present invention provides arrays of oligonucleotide probes immobilized in microfabricated patterns on silica chips for analyzing molecular interactions of biological interest.

The invention therefore relates to diverse fields impacted by the nature of molecular interaction, including chemistry, biology, medicine, and medical diagnostics.

Oligonucleotide probes have long been used to detect complementary nucleic acid sequences in a nucleic acid of interest (the "target" nucleic acid). In some assay formats, the oligonucleotide probe is tethered, i.e., by covalent attachment, to a solid support, and arrays of oligonucleotide probes immobilized on solid supports have been used to detect specific nucleic acid sequences in a target nucleic acid.

See, e.g., PCT patent publication Nos. WO 89/10977 and 89/11548. Others have proposed the use of large numbers of oligonucleotide probes to provide the complete nucleic acid sequence of a target nucleic acid but failed to provide an enabling method for using arrays of immobilized probes for this purpose. See U.S. Patent Nos. 5,202,231 and 5,002,867 and PCT patent publication No. WO 93/17126. See U.S. Patent No. 5,143,854 and PCT patent publication Nos. WO 90/15070 and 92/10092, each of which is incorporated herein by reference. Microfabricated arrays of large numbers of oligonucleotide probes, called "DNA chips" offer great promise for a wide variety of applications. New methods and reagents are required to realize this promise, and the present invention helps meet that need.

6.8.2. General Strategies

The invention provides several strategies employing immobilized arrays of probes for comparing a reference sequence of known sequence with a target sequence showing substantial similarity with the reference sequence, but differing in the presence of, e.g.,

mutations. In a first embodiment, the invention provides a tiling strategy employing an array of immobilized oligonucleotide probes comprising at least two sets of probes. A first probe set comprises a plurality of probes, each probe comprising a segment of at least three nucleotides exactly complementary to a subsequence of the reference sequence, the segment including at least one interrogation position complementary to a corresponding nucleotide in the reference sequence. A second probe set comprises a corresponding probe for each probe in the first probe set, the corresponding probe in the second probe set being identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least three nucleotides thereof that includes the at least one interrogation position, except that the at least one interrogation position is occupied by a different nucleotide in each of the two corresponding probes from the first and second probe sets. The probes in the first probe set have at least two interrogation positions corresponding to two contiguous nucleotides in the reference sequence. One interrogation position corresponds to one of the contiguous nucleotides, and the other interrogation position to the other.

In a second embodiment, the invention provides a tiling strategy employing an array comprising four probe sets. A first probe set comprises a plurality of probes, each probe comprising a segment of at least three nucleotides exactly complementary to a subsequence of the reference sequence, the segment including at least one interrogation position complementary to a corresponding nucleotide in the reference sequence. Second, third and fourth probe sets each comprise a corresponding probe for each probe in the first probe set.

The probes in the second, third and fourth probe sets are identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least three nucleotides thereof that includes the at least one interrogation position, except that the at least one interrogation position is occupied by a different nucleotide in each of the four corresponding probes from the four probe sets. The first probe set often has at least 100 interrogation positions corresponding to 100 contiguous nucleotides in the reference sequence. Sometimes the first probe set has an interrogation position corresponding to every nucleotide in the reference sequence. The segment of complementarity within the probe set is usually about 9-21 nucleotides. Although probes may contain leading or

trailing sequences in addition to the 9-21 sequences, many probes consist exclusively of a 9-21 segment of complementarity.

In a third embodiment, the invention provides immobilized arrays of probes tiled for multiple reference sequences. one such array comprises at least one pair of first and second probe groups, each group comprising first and second sets of probes as defined in the first embodiment. Each probe in the first probe set from the first group is exactly complementary to a subsequence of a first reference sequence, and each probe in the first probe set from the second group is exactly complementary to a subsequence of a second reference sequence.

Thus, the first group of probes are tiled with respect to a first reference sequence and the second group of probes with respect to a second reference sequence. Each group of probes can also include third and fourth sets of probes as defined in the second embodiment. In some arrays of this type, the second reference sequence is a mutated form of the first reference sequence.

In a fourth embodiment, the invention provides arrays for block tiling. Block tiling is a species of the general tiling strategies described above. The usual unit of a block tiling array is a group of probes comprising a wildtype probe, a first set of three mutant probes and a second set of three mutant probes. The wildtype probe comprises a segment of at least three nucleotides exactly complementary to a subsequence of a reference sequence. The segment has at least first and second interrogation positions corresponding to first and second nucleotides in the reference sequence. The probes in the first set of three mutant probes are each identical to a sequence comprising the wildtype probe or a subsequence of at least three nucleotides thereof including the first and second interrogation positions, except in the first interrogation position, which is occupied by a different nucleotide in each of the three mutant probes and the wildtype probe. The probes in the second set of three mutant probes are each identical to a sequence comprising the wildtype probes or a subsequence of at least three nucleotides thereof including the first and second interrogation positions, except in the second interrogation position, which is occupied by a different nucleotide in each of the three mutant probes and the wildtype probe.

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referred to as deletion tiling, the first and second subsequences are separated by one or two nucleotides in the reference sequence.

In an eighth embodiment, the invention provides arrays of probes for multiplex tiling. Multiplex tiling is a strategy, in which the identity of two nucleotides in a target sequence is determined from a comparison of the hybridization intensities of four probes, each having two interrogation positions. Each of the probes comprising a segment of at least 7 nucleotides that is exactly complementary to a subsequence from a reference sequence, except that the segment may or may not be exactly complementary at two interrogation positions. The nucleotides occupying the interrogation positions are selected by the following rules: (1) the first interrogation position is occupied by a different nucleotide in each of the four probes, (2) the second interrogation position is occupied by a different nucleotide in each of the four probes, (3) in first and second probes, the segment is exactly complementary to the subsequence, except at no more than one of the interrogation positions, (4) in third and fourth probes, the segment is exactly complementary to the subsequence, except at both of the interrogation positions.

In a ninth embodiment, the invention provides arrays of immobilized probes including helper mutations. Helper mutations are useful for, e.g., preventing self-annealing of probes having inverted repeats. In this strategy, the identity of a nucleotide in a target sequence is usually determined from a comparison of four probes. A first probe comprises a segment of at least 7 nucleotides exactly complementary to a subsequence of a reference sequence except at one or two positions, the segment including an interrogation position not at the one or two positions. The one or two positions are occupied by helper mutations.

Second, third and fourth mutant probes are each identical to a sequence comprising the wildtype probe or a subsequence thereof including the interrogation position and the one or two positions, except in the interrogation position, which is occupied by a different nucleotide in each of the four probes.

In a tenth embodiment, the invention provides arrays of probes comprising at least two probe sets, but lacking a probe set comprising probes that are perfectly matched to a reference sequence. Such arrays are usually employed in methods in which both reference

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In a twelfth embodiment, the invention provides arrays of immobilized probes in which the probes are designed to tile a reference sequence from a human immunodeficiency virus.

Reference sequences from either the reverse transcriptase gene or protease gene of HIV are of particular interest. Some chips further comprise arrays of probes tiling a reference sequence from a 16S RNA or DNA encoding the 16S RNA from a pathogenic microorganism. The invention further provides methods of using such arrays in analyzing a HIV target sequence. The methods are particularly useful where the target sequence has a substituted nucleotide relative to the reference sequence in at least one position, the substitution conferring resistance to a drug use in treating a patient infected with a HIV virus. The methods reveal the existence of the substituted nucleotide. The methods are also particularly useful for analyzing a mixture of undetermined proportions of first and second target sequences from different HIV variants. The relative specific binding of probes indicates the proportions of the first and second target sequences.

In a thirteenth embodiment, the invention provides arrays of probes tiled based on reference sequence from a CFTR gene. A preferred array comprises at least a group of probes comprising a wildtype probe, and five sets of three mutant probes. The wildtype probe is exactly complementary to a subsequence of a reference sequence from a cystic fibrosis gene, the segment having at least five interrogation positions corresponding to five contiguous nucleotides in the reference sequence. The probes in the first set of three mutant probes are each identical to the wildtype probe, except in a first of the five interrogation positions, which is occupied by a different nucleotide in each of the three mutant probes and the wildtype probe. The probes in the second set of three mutant probes are each identical to the wildtype probe, except in a second of the five interrogation positions, which is occupied by a different nucleotide in each of the three mutant probes and the wildtype probe. The probes in the third set of three mutant probes are each identical to the wildtype probe, except in a third of the five interrogation positions, which is occupied by a different nucleotide in each of the three mutant probes and the wildtype probe. The probes in the fourth set of three mutant probes are each identical to the wildtype probe, except in a fourth of the five interrogation positions, which is occupied by

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The strategy usually entails sampling each nucleotide of interest in a target sequence several times, thereby achieving a high degree of confidence in its identity. This level of confidence is further increased by sampling of adjacent nucleotides in the target sequence to nucleotides of interest.

Some advantage of the use of only a small proportion of all possible probes of a given length include: (i) each position in the array is highly informative, whether or not hybridization occurs; (ii) nonspecific hybridization is minimized; (iii) it is straightforward to correlate hybridization differences with sequence differences, particularly with reference to the hybridization pattern of a known standard; and (iv) the ability to address each probe independently during synthesis, using high resolution photolithography, allows the array to be designed and optimized for any sequence. For example the length of any probe can be varied independently of the others.

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divergence is anticipated between target sequences, more than one reference sequence is selected. Combinations of wildtype and mutant reference sequences are employed in several applications of the tiling strategy.

6.8.3.1.2. Chip Design

6.8.3.1.2.1. Basic Tiling Strategy

The basic tiling strategy provides an array of immobilized probes for analysis of target sequences showing a high degree of sequence identity to one or more selected reference sequences. The strategy is first illustrated for an array that is subdivided into four probe sets, although it will be apparent that in some situations, satisfactory results are obtained from only two probe sets. A first probe set comprises a plurality of probes exhibiting perfect complementarity with a selected reference sequence. The perfect complementarity usually exists throughout the length of the probe. However, probes having a segment or segments of perfect complementarity that is/are flanked by leading or trailing sequences lacking complementarity to the reference sequence can also be used. Within a segment of complementarity, each probe in the first probe set has at least one interrogation position that corresponds to a nucleotide in the reference sequence. That is, the interrogation position is aligned with the corresponding nucleotide in the reference sequence, when the probe and reference sequence are aligned to maximize complementarity between the two. If a probe has more than one interrogation position, each corresponds with a respective nucleotide in the reference sequence. The identity of an interrogation position and corresponding nucleotide in a particular probe in the first probe set cannot be determined simply by inspection of the probe in the first set. As will become apparent, an interrogation position and corresponding nucleotide is defined by the comparative structures of probes in the first probe set and corresponding probes from additional probe sets.

In principle, a probe could have an interrogation position at each position in the segment complementary to the reference sequence. Sometimes, interrogation positions provide more accurate data when located away from the ends of a segment of complementarity. Thus, typically a probe having a segment of complementarity of length x does not contain

more than $x-2$ interrogation positions. Since probes are typically 9-21 nucleotides, and usually all of a probe is complementary, a probe typically has 1-19 interrogation positions. Often the probes contain a single interrogation position, at or near the center of probe.

For each probe in the first set, there are, for purposes of the present illustration, three corresponding probes from three additional probe sets. Thus, there are four probes corresponding to each nucleotide of interest in the reference sequence. Each of the four corresponding probes has an interrogation position aligned with that nucleotide of interest. Usually, the probes from the three additional probe sets are identical to the corresponding probe from the first probe set with one exception. The exception is that at least one (and often only one) interrogation position, which occurs in the same position in each of the four corresponding probes from the four probe sets, is occupied by a different nucleotide in the four probe sets. For example, for an A nucleotide in the reference sequence, the corresponding probe from the first probe set has its interrogation position occupied by a T, and the corresponding probes from the additional three probe sets have their respective interrogation positions occupied by A, C, or G, a different nucleotide in each probe. Of course, if a probe from the first probe set comprises trailing or flanking sequences lacking complementarity to the reference sequences, these sequences need not be present in corresponding probes from the three additional sets. Likewise corresponding probes from the three additional sets can contain leading or trailing sequences outside the segment of complementarity that are not present in the corresponding probe from the first probe set. Occasionally, the probes from the additional three probe set are identical (with the exception of interrogation position(s)) to a contiguous subsequence of the full complementary segment of the corresponding probe from the first probe set. In this case, the subsequence includes the interrogation position and usually differs from the full-length probe only in the omission of one or both terminal nucleotides from the termini of a segment of complementarity.

That is, if a probe from the first probe set has a segment of complementarity of length n , corresponding probes from the other sets will usually include a subsequence of the segment of at least length $n-2$. Thus, the subsequence is usually at least 3, 4, 7, 9, 15, 21, or 25 nucleotides long, most typically, in the range of 9-21 nucleotides. The subsequence should be sufficiently long to allow a probe to hybridize detectably more strongly to a



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The number of probes in the first probe set (and as a consequence the number of probes in additional probe sets) depends on the length of the reference sequence, the number of nucleotides of interest in the reference sequence and the number of interrogation positions per probe. In general, each nucleotide of interest in the reference sequence requires the same interrogation position in the four sets of probes.

In some reference sequences, every nucleotide is of interest. In other reference sequences, only certain portions in which variants (e.g., mutations or polymorphisms) are concentrated are of interest. In other reference sequences, only particular mutations or polymorphisms and immediately adjacent nucleotides are of interest. Usually, the first

probe set has interrogation positions selected to correspond to at least a nucleotide (e.g., representing a point mutation) and one immediately adjacent nucleotide. Usually, the probes in the first set have interrogation positions corresponding to at least 3, 10, 50, 100, 1000, or 20,000 contiguous nucleotides. The probes usually have interrogation positions corresponding to at least 5, 10, 30, 50, 75, 90, 99 or sometimes 100% of the nucleotides in a reference sequence.

Frequently, the probes in the first probe set completely span the reference sequence and overlap with one another relative to the reference sequence. For example, in one common arrangement each probe in the first probe set differs from another probe in that set by the omission of a 3' base complementary to the reference sequence and the acquisition of a 5' base complementary to the reference sequence.

For conceptual simplicity, the probes in a set are usually arranged in order of the sequence in a lane across the chip. A lane contains a series of overlapping probes, which represent or tile across, the selected reference sequence. The components of the four sets of probes are usually laid down in four parallel lanes, collectively constituting a row in the horizontal direction and a series of 4-member columns in the vertical direction. Corresponding probes from the four probe sets (i.e., complementary to the same subsequence of the reference sequence) occupy a column.

Each probe in a lane usually differs from its predecessor in the lane by the omission of a base at one end and the inclusion of additional base at the other end. However, this orderly progression of probes can be interrupted by the inclusion of control probes or omission of probes in certain columns of the array. Such columns serve as controls to orient the chip, or gauge the background, which can include target sequence nonspecifically bound to the chip.

The probes sets are usually laid down in lanes such that all probes having an interrogation position occupied by an A form an A-lane, all probes having an interrogation position occupied by a C form a C-lane, all probes having an interrogation position occupied by a G form a G-lane, and all probes having an interrogation position occupied by a T (or U) form a T lane (or a U lane). Note that in this arrangement there is not a unique

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Although the array of probes is usually laid down in rows and columns as described above, such a physical arrangement of probes on the chip is not essential. Provided that the spatial location of each probe in an array is known, the data from the probes can be collected and processed to yield the sequence of a target irrespective of the physical arrangement of the probes on a chip. In processing the data, the hybridization signals from the respective probes can be reassorted into any conceptual array desired for subsequent data reduction whatever the physical arrangement of probes on the chip.

A range of lengths of probes can be employed in the chips. As noted above, a probe may consist exclusively of a complementary segments, or may have one or more complementary segments juxtaposed by flanking, trailing and/or intervening segments. In the latter situation, the total length of complementary segment(s) is more important than the length of the probe. In functional terms, the complementarity segment(s) of the first probe sets should be sufficiently long to allow the probe to hybridize detectably more strongly to a reference sequence compared with a variant of the reference including a single base mutation at the nucleotide corresponding to the interrogation position of the probe.

Similarly, the complementarity segment(s) in corresponding probes from additional probe sets should be sufficiently long to allow a probe to hybridize detectably more strongly to a variant of the reference sequence having a single nucleotide substitution at the interrogation position relative to the reference sequence. A probe usually has a single complementary segment having a length of at least 3 nucleotides, and more usually at least

5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or bases exhibiting perfect complementarity (other than possibly at the interrogation position(s) depending on the probe set) to the reference sequence. In bridging strategies, where more than one segment of complementarity is present, each segment provides at least three complementary nucleotides to the reference sequence and the combined segments provide at least two segments of three or a total of six complementary nucleotides. As in the other strategies, the combined length of complementary segments is typically from 6-30 nucleotides, and preferably from about 9-21 nucleotides. The two segments are often approximately the same length. Often, the probes (or segment of complementarity within probes) have an odd number of bases, so that an interrogation position can occur in the exact center of the probe.

In some chips, all probes are the same length. Other chips employ different groups of probe sets, in which case the probes are of the same size within a group, but differ between different groups. For example, some chips have one group comprising four sets of probes as described above in which all the probes are 11 mers, together with a second group comprising four sets of probes in which all of the probes are 13 mers. Of course, additional groups of probes can be added.

Thus, some chips contain, e.g., four groups of probes having sizes of 11 mers, 13 mers, 15 mers and 17 mers. Other chips have different size probes within the same group of four probe sets. In these chips, the probes in the first set can vary in length independently of each other. Probes in the other sets are usually the same length as the probe occupying the same column from the first set. However, occasionally different lengths of probes can be included at the same column position in the four lanes. The different length probes are included to equalize hybridization signals from probes irrespective of whether A-T or C-G bonds are formed at the interrogation position.

The length of probe can be important in distinguishing between a perfectly matched probe and probes showing a single- base mismatch with the target sequence. The discrimination is usually greater for short probes. Shorter probes are usually also less susceptible to formation of secondary structures.

However, the absolute amount of target sequence bound, and hence the signal, is greater for larger probes. The probe length representing the optimum compromise between these competing considerations may vary depending on inter alia the GC content of a particular region of the target DNA sequence, secondary structure, synthesis efficiency and cross-hybridization. In some regions of the target, depending on hybridization conditions, short probes (e.g., 11 mers) may provide information that is inaccessible from longer probes (e.g., 19 mers) and vice versa. Maximum sequence information can be read by including several groups of different sized probes on the chip as noted above. However, for many regions of the target sequence, such a strategy provides redundant information in that the same sequence is read multiple times from the different groups of probes. Equivalent information can be obtained from a single group of different sized probes in which the sizes are selected to maximize readable sequence at particular regions of the target sequence. The strategy of customizing probe length within a single group of probe sets minimizes the total number of probes required to read a particular target sequence. This leaves ample capacity for the chip to include probes to other reference sequences.

The invention provides an optimization block which allows systematic variation of probe length and interrogation position to optimize the selection of probes for analyzing a particular nucleotide in a reference sequence. The block comprises alternating columns of probes complementary to the wildtype target and probes complementary to a specific mutation. The interrogation position is varied between columns and probe length is varied down a column.

Hybridization of the chip to the reference sequence or the mutant form of the reference sequence identifies the probe length and interrogation position providing the greatest differential hybridization signal.

The probes are designed to be complementary to either strand of the reference sequence (e.g., coding or non-coding). some chips contain separate groups of probes, one complementary to the coding strand, the other complementary to the noncoding strand. Independent analysis of coding and noncoding strands provides largely redundant information.

However, the regions of ambiguity in reading the coding strand are not always the same as those in reading the noncoding strand. Thus, combination of the information from coding and noncoding strands increases the overall accuracy of sequencing.

Some chips contain additional probes or groups of probes designed to be complementary to a second reference sequence.

The second reference sequence is often a subsequence of the first reference sequence bearing one or more commonly occurring mutations or interstrain variations. The second group of probes is designed by the same principles as described above except that the probes exhibit complementarity to the second reference sequence. The inclusion of a second group is particularly useful for analyzing short subsequences of the primary reference sequence in which multiple mutations are expected to occur within a short distance commensurate with the length of the probes (i.e., two or more mutations within 9 to 21 bases). Of course, the same principle can be extended to provide chips containing groups of probes for any number of reference sequences. Alternatively, the chips may contain additional probe(s) that do not form part of a tiled array as noted above, but rather serves as probe(s) for a conventional reverse dot blot. For example, the presence of mutation can be detected from binding of a target sequence to a single oligomeric probe harboring the mutation. Preferably, an additional probe containing the equivalent region of the wildtype sequence is included as a control.

The chips are read by comparing the intensities of labelled target bound to the probes in an array.

Specifically, a comparison is performed between each lane of probes (e.g., A, C, G and T lanes) at each columnar position (physical or conceptual). For a particular columnar position, the lane showing the greatest hybridization signal is called as the nucleotide present at the position in the target sequence corresponding to the interrogation position in the probes. The corresponding position in the target sequence is that aligned with the interrogation position in corresponding probes when the probes and target are aligned to maximize complementarity. Of the four probes in a column, only one can exhibit a perfect match to the target sequence whereas the others usually exhibit at least a one base pair

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is mismatched with respect to the reference sequence, and a corresponding decrease in the signal from the probe which is matched with the reference sequence signal the presence of a mutant strain in the mixture. The extent in shift in hybridization signals of the probes is related to the proportion of a target sequence in the mixture. Shifts in relative hybridization signals can be quantitatively related to proportions of reference and mutant sequence by prior calibration of the chip with seeded mixtures of the mutant and reference sequences. By this means, a chip can be used to detect variant or mutant strains constituting as little as 1, 5, 20, or 25 % of a mixture of stains.

Similar principles allow the simultaneous analysis of multiple target sequences even when none is identical to the reference sequence. For example, with a mixture of two target sequences bearing first and second mutations, there would be a variation in the hybridization patterns of probes having interrogation positions corresponding to the first and second mutations relative to the hybridization pattern with the reference sequence. At each position, one of the probes having a mismatched interrogation position relative to the reference sequence would show an increase in hybridization signal, and the probe having a matched interrogation position relative to the reference sequence would show a decrease in hybridization signal. Analysis of the hybridization pattern of the mixture of mutant target sequences, preferably in comparison with the hybridization pattern of the reference sequence, indicates the presence of two mutant target sequences, the position and nature of the mutation in each strain, and the relative proportions of each strain.

In a variation of the above method, the different components in a mixture of target sequences are differentially labelled before being applied to the array. For example, a variety of fluorescent labels emitting at different wavelength are available. The use of differential labels allows independent analysis of different targets bound simultaneously to the array. For example, the methods permit comparison of target sequences obtained from a patient at different stages of a disease.

6.8.3.1.2.2. Omission of Probes

The general strategy outlined above employs four probes to read each nucleotide of interest in a target sequence. One probe (from the first probe set) shows a perfect match to the reference sequence and the other three probes (from the second, third and fourth probe sets) exhibit a mismatch with the reference sequence and a perfect match with a target sequence bearing a mutation at the nucleotide of interest.

The provision of three probes from the second, third and fourth probe sets allows detection of each of the three possible nucleotide substitutions of any nucleotide of interest.

However, in some reference sequences or regions of reference sequences, it is known in advance that only certain mutations are likely to occur. Thus, for example, at one site it might be known that an A nucleotide in the reference sequence may exist as a T mutant in some target sequences but is unlikely to exist as a C or G mutant. Accordingly, for analysis of this region of the reference sequence, one might include only the first and second probe sets, the first probe set exhibiting perfect complementarity to the reference sequence, and the second probe set having an interrogation position occupied by an invariant A residue (for detecting the T mutant). In other situations, one might include the first, second and third probes sets (but not the fourth) for detection of a wildtype nucleotide in the reference sequence and two mutant variants thereof in target sequences. In some chips, probes that would detect silent mutations (i.e., not affecting amino acid sequence) are omitted.

In some chips, the probes from the first probe set are omitted corresponding to some or all positions of the reference sequences. Such chips comprise at least two probe sets. The first probe set has a plurality of probes. Each probe comprises a segment exactly complementary to a subsequence of a reference sequence except in at least one interrogation position. A second probe set has a corresponding probe for each probe in the first probe set.

The corresponding probe in the second probe set is identical to a sequence comprising the corresponding probe from the first probe set or a subsequence thereof that includes the at least one (and usually only one) interrogation position except that the at least one

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When the chips comprise four probe sets, as discussed supra, and the probe sets are laid down in four lanes, an A-lane, a C-lane, a G-lane and a T or U-lane, the probe having a segment exhibiting perfect complementarity to a reference sequence varies between the four lanes from one column to another. This does not present any significant difficulty in computer analysis of the data from the chip. However, visual inspection of the hybridization pattern of the chip is sometimes facilitated by provision of an extra lane of probes, in which each probe has a segment exhibiting perfect complementarity to the reference sequence. This segment is identical to a segment from one of the probes in the other four lanes (which lane depending on the column position). The extra lane of probes (designated the wildtype lane) hybridizes to a target sequence at all nucleotide positions except those in which deviations from the reference sequence occurs. The hybridization pattern of the wildtype lane thereby provides a simple visual indication of mutations.

nucleotides complementary to the nucleotides occupying corresponding positions in the reference sequence when the possible substitutions have occurred.

6.8.3.1.2.5. Block Tiling

As noted in the discussion of the general tiling strategy, a probe in the first probe set sometimes has more than one interrogation position. In this situation, a probe in the first probe set is sometimes matched with multiple groups of at least one, and usually, three additional probe sets. Three additional probe sets are used to allow detection of the three possible nucleotide substitutions at any one position. If only certain types of substitution are likely to occur (e.g., transitions), only one or two additional probe sets are required (analogous to the use of probes in the basic tiling strategy). To illustrate for the situation where a group comprises three additional probe sets, a first such group comprises second, third and fourth probe sets, each of which has a probe corresponding to each probe in the first probe set. The corresponding probes from the second, third and fourth probes sets differ from the corresponding probe in the first set at a first of the interrogation positions. Thus, the relative hybridization signals from corresponding probes from the first, second, third and fourth probe sets indicate the identity of the nucleotide in a target sequence corresponding to the first interrogation position. A second group of three probe sets (designated fifth, sixth and seventh probe sets), each also have a probe corresponding to each probe in the first probe set. These corresponding probes differ from that in the first probe set at a second interrogation position. The relative hybridization signals from corresponding probes from the first, fifth, sixth, and seventh probe sets indicate the identity of the nucleotide in the target sequence corresponding to the second interrogation position. As noted above, the probes in the first probe set often have seven or more interrogation positions. If there are seven interrogation positions, there are seven groups of three additional probe sets, each group of three probe sets serving to identify the nucleotide corresponding to one of the seven interrogation positions.

Each block of probes allows short regions of a target sequence to be read. For example, for a block of probes having seven interrogation positions, seven nucleotides in the target sequence can be read. Of course, a chip can contain any number of blocks depending on how many nucleotides of the target are of interest. The hybridization signals for each

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6.8.3.1.2.6. Multiplex Tiling

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...ed by a different nucleotide in each of the
...gnated the first and second probes, the segment
...ence sequence except at not more than one of the two
The second interrogation
four probes. In two words, one of the interrogation positions is occupied by a
is exactly complementary to the corresponding nucleotide from the reference
interrogation position may or may not be so occupied. In the other
nucleotide probes, designated the third and fourth probes, the segment is exactly
sequence to the reference sequence except that both interrogation positions are
two by nucleotides which are noncomplementary to the respective corresponding
nucleotides in the reference sequence.

There are number of ways of satisfying these conditions depending on whether the two nucleotides in the reference sequence corresponding to the two interrogation positions are the same or different. If these two nucleotides are different in the reference sequence (probability $3/4$), the conditions are satisfied by each of the two interrogation positions being occupied by the same nucleotide in any given probe. For example, in the first probe, the two interrogation positions would both be A, in the second probe, both would be C, in the third probe, each would be G, and in the fourth probe each would be T or U. If the two nucleotides in the reference sequence corresponding to the two interrogation positions are different, the conditions noted above are satisfied by each of the interrogation positions in any one of the four probes being occupied by complementary nucleotides. For example, in the first probe, the interrogation positions could be occupied by A and T, in the second probe by C and G, in the third probe by G and C and in the four probe, by T and A.

When the four probes are hybridized to a target that is the same as the reference sequence or differs from the reference sequence at one (but not both) of the interrogation positions, two of the four probes show a double-mismatch with the target and two probes show a single mismatch. The identity of probes showing these different degrees of mismatch can be determined from the different hybridization signals.

From the identity of the probes showing the different degrees of mismatch, the nucleotides occupying both of the interrogation positions in the target sequence can be deduced.

For ease of illustration, the multiplex strategy has been initially described for the situation where there are two nucleotides of interest in a reference sequence and only four probes in an array. Of course, the strategy can be extended to analyze any number of nucleotides in a target sequence by using additional probes. In one variation, each pair of interrogation positions is read from a unique group of four probes. In a block variation, different groups of four probes exhibit the same segment of complementarity with the reference sequence, but the interrogation positions move within a block.

The block and standard multiplex tiling variants can of course be used in combination for different regions of a reference sequence. Either or both variants can also be used in combination with any of the other tiling strategies described.

6.8.3.1.2.7. Helper Mutations

Occasionally small regions of a reference sequence give a low hybridization signal as a result of annealing of probes.

The self-annealing reduces the amount of probe effectively available for hybridizing to the target. Although such regions of the target are generally small and the reduction of hybridization signal is usually not so substantial as to obscure the sequence of this region, this concern can be avoided by the use of probes incorporating helper mutations.

The helper mutation(s) serve to break-up regions of internal complementarity within a probe and thereby prevent annealing.

Usually, one or two helper mutations are quite sufficient for this purpose. The inclusion of helper mutations can be beneficial in any of the tiling strategies noted above. In general each probe having a particular interrogation position has the same helper mutation(s). Thus, such probes have a segment in common which shows perfect complementarity with a reference sequence, except that the segment contains at least one helper mutation (the same in each of the probes) and at least one interrogation position (different in all of the probes). For example, in the basic tiling strategy, a probe from the first probe set comprises a segment containing an interrogation position and showing perfect

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DISCUSSION

For example, consider an array of five pooled probes each having the general structure outlined above. Three of these pooled probes have an interrogation position that aligns with the same nucleotide in the reference sequence and are used to read that nucleotide. A different combination of three probes have an interrogation position that aligns with a different nucleotide in the reference sequence. Comparison of these three probe intensities allows analysis of this second nucleotide. Still another combination of three pooled probes from the set of five have an interrogation position that aligns with a third nucleotide in the reference sequence and these probes are used to analyze that nucleotide. Thus, three nucleotides in the reference sequence are fully analyzed from only five pooled probes. By comparison, the basic tiling strategy would require 12 probes for a similar analysis.

Consider the use of three such pooled probes for analyzing a target sequence, of which one position may contain any single base substitution to the reference sequence (i.e, there are four possible target sequences to be distinguished).

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The use of deletion or bridging probes is quite general. These probes can be used in any of the tiling strategies of the invention. As well as offering superior discrimination, the use of deletion or bridging strategies is advantageous for certain probes to avoid self-hybridization (either within a probe or between two probes of the same sequence)

6.8.3.1.3. Preparation of Target Samples

The target polynucleotide, whose sequence is to be determined, is usually isolated from a tissue sample. If the target is genomic, the sample may be from any tissue (except exclusively red blood cells). For example, whole blood, peripheral blood lymphocytes or PBMC, skin, hair or semen are convenient sources of clinical samples. These sources are also suitable if the target is RNA. Blood and other body fluids are also a convenient source for isolating viral nucleic acids. If the target is mRNA, the sample is obtained from a tissue in which the mRNA is expressed. If the polynucleotide in the sample is RNA, it is usually reverse transcribed to DNA. DNA samples or cDNA resulting from reverse transcription are usually amplified, e.g., by PCR. Depending on the selection of primers and amplifying enzyme(s), the amplification product can be RNA or DNA.

Paired primers are selected to flank the borders of a target polynucleotide of interest. More than one target can be simultaneously amplified by multiplex PCR in which multiple paired primers are employed. The target can be labelled at one or more nucleotides during or after amplification. For some target polynucleotides (depending on size of sample), e.g., episomal DNA, sufficient DNA is present in the tissue sample to dispense with the amplification step.

When the target strand is prepared in single-stranded form as in preparation of target RNA, the sense of the strand should of course be complementary to that of the probes on the chip. This is achieved by appropriate selection of primers.

The target is preferably fragmented before application to the chip to reduce or eliminate the formation of secondary structures in the target. The average size of targets segments following hybridization is usually larger than the size of probe on the chip.

6.8.3.2. Modes Of Practicing The Invention

In an exemplary method, light is shone through a mask to activate functional (for oligonucleotides, typically an -OH) groups protected with a photoremovable protecting group on a surface of a solid support. After light activation, a nucleoside building block, itself protected with a photoremovable protecting group (at the 5'-OH), is coupled to the activated areas of the support. The process can be repeated, using different masks or mask orientations and building blocks, to prepare very dense arrays of many different oligonucleotide probes. New methods for the combinatorial chemical synthesis of peptide, polycarbamate, and oligonucleotide arrays have recently been reported (see Fodor et al., 1991, Science 251: 767-773; Cho et al., 1993, Science 261: 1303-1305; and Southern et al., 1992, Genomics 13: 1008-10017, each of which is incorporated herein by reference). These arrays, or biological chips (see Fodor et al., 1993, Nature 364: 555-556, incorporated herein by reference), harbor specific chemical compounds at precise locations in a high-density, information rich format, and are a powerful tool for the study of biological recognition processes. A particularly exciting application of the array technology is in the field of DNA sequence analysis. The hybridization pattern of a DNA target to an array of shorter oligonucleotide probes is used to gain primary structure information of the DNA target. This format has important applications in sequencing by hybridization, DNA diagnostics and in elucidating the thermodynamic parameters affecting nucleic acid recognition.

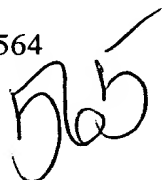
Conventional DNA sequencing technology is a laborious procedure requiring electrophoretic size separation of labeled DNA fragments. An alternative approach, termed Sequencing By Hybridization (SBH), has been proposed (Lysov et al., 1988, Dokl. Akad. Nauk SSSR 303:1508-1511; Bains et al., 1988, J. Theor. Biol. 135:303-307; and Drmanac et al., 1989, Genomics 4:114-128, incorporated herein by reference). This method uses a set of short oligonucleotide probes of defined sequence to search for complementary sequences on a longer target strand of DNA. The hybridization pattern is used to reconstruct the target DNA sequence. It is envisioned that hybridization analysis of large numbers of probes can be used to sequence long stretches of DNA. In immediate applications of this hybridization methodology, a small number of probes can be used to interrogate local DNA sequence.

Hybridization methodology can be carried out by attaching target DNA to a surface. The target is interrogated with a set of oligonucleotide probes, one at a time (see Strezoska et al., 1991, Proc. Natl. Acad. Sci. USA 88:10089-10093, and Drmanac et al., 1993, Science 260:1649-1652, each of which is incorporated herein by reference). This approach can be implemented with well established methods of immobilization and hybridization detection, but involves a large number of manipulations. For example, to probe a sequence utilizing a full set of octanucleotides, tens of thousands of hybridization reactions must be performed. Alternatively, SBH can be carried out by attaching probes to a surface in an array format where the identity of the probes at each site is known. The target DNA is then added to the array of probes.

The hybridization pattern determined in a single experiment directly reveals the identity of all complementary probes.

As noted above, a preferred method of oligonucleotide probe array synthesis involves the use of light to direct the synthesis of oligonucleotide probes in high-density, miniaturized arrays. Photolabile 5'-protected N-acyl-deoxynucleoside phosphoramidites, surface linker chemistry, and versatile combinatorial synthesis strategies have been developed for this technology. Matrices of spatially-defined oligonucleotide probes have been generated, and the ability to use these arrays to identify complementary sequences has been demonstrated by hybridizing fluorescent labeled oligonucleotides to the DNA chips produced by the methods. The hybridization pattern demonstrates a high degree of base specificity and reveals the sequence of oligonucleotide targets.

The surface of a solid support modified with photolabile protecting groups is illuminated through a photolithographic mask, yielding reactive hydroxyl groups in the illuminated regions. A 3'-O-phosphoramidite activated deoxynucleoside (protected at the 5'-hydroxyl with a photolabile group) is then presented to the surface and coupling occurs at sites that were exposed to light. Following capping, and oxidation, the substrate is rinsed and the surface illuminated through a second mask, to expose additional hydroxyl groups for coupling. A second 5'-protected, 3'-O-phosphoramidite activated deoxynucleoside is



presented to the surface. The selective photodeprotection and coupling cycles are repeated until the desired set of products is obtained.

Light directed chemical synthesis lends itself to highly efficient synthesis strategies which will generate a maximum number of compounds in a minimum number of chemical steps.

To carry out hybridization of DNA targets to the probe arrays, the arrays are mounted in a thermostatically controlled hybridization chamber. Fluorescein labeled DNA targets are injected into the chamber and hybridization is allowed to proceed for 5 min to 24 hr. The surface of the matrix is scanned in an epifluorescence microscope (Zeiss Axioscop 20) equipped with photon counting electronics using 50 -100 uW of 488 nm excitation from an Argon ion laser (Spectra Physics Model 2020). Measurements may be made with the target solution in contact with the probe matrix or after washing. Photon counts are stored and image files are presented after conversion to an eight bit image format.

Arrays of oligonucleotides can be efficiently generated by light-directed synthesis and can be used to determine the identity of DNA target sequences. Because combinatorial strategies are used, the number of compounds increases exponentially while the number of chemical coupling cycles increases only linearly. For example, synthesizing the complete set of 4^8 (65,536) octanucleotides will add only four hours to the synthesis for the 16 additional cycles.

Furthermore, combinatorial synthesis strategies can be implemented to generate arrays of any desired composition.

For example, because the entire set of dodecamers (4^{12}) can be produced in 48 photolysis and coupling cycles (b^n compounds requires $b \times n$ cycles), any subset of the dodecamers (including any subset of shorter oligonucleotides) can be constructed with the correct lithographic mask design in 48 or fewer chemical coupling steps. In addition, the number of compounds in an array is limited only by the density of synthesis sites and the overall array size. Recent experiments have demonstrated hybridization to probes synthesized in 25 um sites. At this resolution, the entire set of 65,536 octanucleotides can be placed in an

array measuring 0.64 cm square, and the set of 1,048,576 dodecanucleotides requires only a 2.56 cm array.

Genome sequencing projects will ultimately be limited by DNA sequencing technologies. Current sequencing methodologies are highly reliant on complex procedures and require substantial manual effort. Sequencing by hybridization has the potential for transforming many of the manual efforts into more efficient and automated formats. Light-directed synthesis is an efficient means for large scale production of miniaturized arrays for SBH. The oligonucleotide arrays are not limited to primary sequencing applications. Because single base changes cause multiple changes in the hybridization pattern, the oligonucleotide arrays provide a powerful means to check the accuracy of previously elucidated DNA sequence, or to scan for changes within a sequence. In the case of octanucleotides, a single base change in the target DNA results in the loss of eight complements, and generates eight new complements. Matching of hybridization patterns may be useful in resolving sequencing ambiguities from standard gel techniques, or for rapidly detecting DNA mutational events. The potentially very high information content of light-directed oligonucleotide arrays will change genetic diagnostic testing. Sequence comparisons of hundreds to thousands of different genes will be assayed simultaneously instead of the current one, or few at a time format. Custom arrays can also be constructed to contain genetic markers for the rapid identification of a wide variety of pathogenic organisms.

Oligonucleotide arrays can also be applied to study the sequence specificity of RNA or protein-DNA interactions.

Experiments can be designed to elucidate specificity rules of non Watson-Crick oligonucleotide structures or to investigate the use of novel synthetic nucleoside analogs for antisense or triple helix applications. Suitably protected RNA monomers may be employed for RNA synthesis. The oligonucleotide arrays should find broad application deducing the thermodynamic and kinetic rules governing formation and stability of oligonucleotide complexes.

Other than the use of photoremovable protecting groups, the nucleoside coupling chemistry is very similar to that used routinely today for oligonucleotide synthesis.

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7 PLANT GENE EXPRESSION

7. General Considerations

The present invention provides a chimeric recombinant DNA molecule comprising: a plurality of DNA sequences, each of which comprises a plant-functional promoter linked to a coding region, which encodes a virus-associated coat protein, wherein said DNA sequences are preferably linked in-tandem so that they are expressed in virus- susceptible plant cells transformed with said recombinant DNA molecule to impart resistance to said viruses; as well as methods for transforming plants with the chimeric constructs and for selecting plants which express at least one of said DNA sequences imparting viral resistance

A method for making a genetically modified plant comprising regenerating a whole plant from a plant cell that has been transfected with DNA sequences comprising a first gene whose expression results in an altered plant phenotype linked to a transiently active promoter, the gene and promoter being separated by a blocking sequence flanked on either side by specific excision sequences, a second gene that encodes a recombinase specific for the specific excision sequences linked to a repressible promoter, and a third gene that encodes the repressor specific for the repressible promoter. Also a method for making a genetically modified hybrid plant by hybridizing a first plant regenerated from a plant cell that has been transfected with DNA sequences comprising a first gene whose expression results in an altered plant phenotype linked to a transiently active promoter, the gene and promoter being separated by a blocking sequence flanked on either side by specific excision sequences to a second plant regenerated from a second plant cell that has been transfected with DNA sequences comprising a second gene that encodes a recombinase specific for the specific excision sequences linked to a promoter that is active during seed germination, and growing a hybrid plant from the hybrid seed. Plant cells, plant tissues, plant seed and whole plants containing the above DNA sequences are also claimed.

The present invention is also directed to a DNA construct formed from a fusion gene which includes a trait DNA molecule and a silencer DNA molecule. The trait DNA molecule has a length that is insufficient to impart a desired trait to plants transformed

with the trait DNA molecule. The silencer DNA molecule is operatively coupled to the trait DNA molecule with the trait and silencer DNA molecules collectively having sufficient length to impart the trait to plants transformed with the DNA construct. Expression systems, host cells, plants, and plant seeds containing the DNA construct are disclosed. The present invention is also directed to imparting multiple traits to a plant.

The present invention is also directed to methods of introgressing one or more desired quantitative traits into a plant comprising screening one or more restriction fragment length polymorphisms (RFLP) for association with desired quantitative traits (QT), selecting one or more RFLP's showing association with the desired QT's, developing a mathematical model based on the magnitude of the association of RFLP(s) to predict the degree of expression of the desired QT's, and using the thus-selected RFLP(s) and the mathematical model in a plant breeding program to predict the degree of introgression and expression of the desired QT's in plant progeny.

A method for producing one of the following proteins in transgenic monocot plant cells is disclosed: (i) mature, glycosylated α_1 -antitrypsin (AAT) having the same N-terminal amino acid sequence as mature AAT produced in humans and a glycosylation pattern which increases serum halflife substantially over that of mature non-glycosylated AAT; (ii) mature, glycosylated antithrombin III (ATIII) having the same N-terminal amino acid sequence as mature ATIII produced in humans; (iii) mature human serum albumin (HSA) having the same N-terminal amino acid sequence as mature HSA produced in humans and having the folding pattern of native mature HSA as evidenced by its bilirubin-binding characteristics; and (iv) mature, active subtilisin BPN' (BPN') having the same N-terminal amino acid sequence as BPN' produced in *Bacillus*. Monocot plants cells are transformed with a chimeric gene which includes a DNA coding sequence encoding a fusion protein having an (i) N-terminal moiety corresponding to a rice α -amylase signal sequence peptide and, (iii) immediately adjacent the C-terminal amino acid of said peptide, a protein moiety corresponding to the mature protein to be produced.

A process for commercially propagating plants by tissue culture in such a way as both to conserve desired plant morphology and to transform the plant with respect to one or more desired genes. The method includes the steps of (a) creating an Agrobacterium

vector containing the gene sequence desired to be transferred to the propagated plant, preferably together with a marker gene; (b) taking one or more petiole explants from a mother plant and inoculating them with the Agrobacterium vector; (c) conducting callus formation in the petiole sections in culture, in the dark; and (d) culturing the resulting callus in growth medium containing a benzylamino growth regulator such as benzylaminopurine or, most preferably, benzylaminopurine-riboside. Additional optional growth regulators including auxins and cytokinins (indole butyric acid, benzylamine, benzyladenine, benzylaminopurine, alpha naphthylacetic acid and others known in the art) may also be present. Preferably, the petiole tissue is taken from *Pelargonium x domesticum* and the Agrobacterium vector contains an antisense gene for ACC synthase or ACC oxidase to prevent ACC synthase or ACC oxidase expression and, in turn, the ethylene formation for which these enzymes are precursors.

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The present invention also relates to:

- (i) the production of mature proteins in plant cells, and in particular, to the production of proteins in mature secreted form.
- (i) the development of techniques for the commercial production of transgenic plants.

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members of the plant potyvirus group which were at one time classified as distinct virus types, but are presently classified as different strains of the same virus; zucchini yellow mosaic virus (ZYMV); potato virus Y; tobacco etch and many others.

These viruses consist of flexuous, filamentous particles of dimensions approximately 780 X 12 nanometers. The viral particles contain a single-stranded RNA genome containing about 10,000 nucleotides of positive (+, coding, or sense) polarity. Translation of the RNA genome of potyviruses shows that the RNA encodes a single Large polyprotein of about 330 kD. This polyprotein contains several proteins, one of which is a 49kD protease that is specific for the cleavage of the polyprotein into at least six (6) other peptides. These proteins can be found in the infected plant cell and form the necessary components for viral replication. One of the proteins contained within this polyprotein is a 35kD capsid or coat protein which coats and protects the viral RNA from degradation. Another protein is the nuclear inclusion protein, also referred to as replicase, which is believed to function in the replication of the viral RNA. In the course of a potyviral infection, the replicase protein (60 kDa, also referred to as the nuclear inclusion B protein) and the protease protein (50kDa, also referred to as the nuclear inclusion I or nuclear inclusion A protein) are posttranslationally transported across the nuclear membrane into the nucleus of the plant cell at the later stages of viral infection and accumulate to high levels.

Generally, the coat protein gene is located at the 3'-end of the RNA, just prior to a stretch of terminal adenine nucleotide residues (200 to 300 bases). The location of the 49 Kd protease gene appears to be conserved in these viruses. In the tobacco etch virus, the protease cleavage site has been determined to be the dipeptide Gln-Ser, Gln-Gly or Gln-Ala. Conservation of these dipeptides as the cleavage sites in these viral polyproteins is apparent from the sequences of the above-listed potyviruses.

Expression of the coat protein genes from tobacco mosaic virus, alfalfa mosaic virus, cucumber mosaic virus, and potato virus X, among others, in transgenic plants has resulted in plants which are resistant to infection by the respective virus. Some evidence of heterologous protection has also been reported. For example, Namba et al., Phytopathology, 82, 940 (1992) report that expression of coat protein genes from

watermelon mosaic virus-2 or zucchini yellow mosaic virus in transgenic tobacco plants conferred protection against six other potyviruses: bean yellow mosaic virus, potato virus Y, pea mosaic virus, clover yellow vein virus, pepper mottle virus and tobacco etch virus. Stark et al., *Biotechnology*, 1, 1257 (1989) report that expression of the potyvirus soybean mosaic virus in transgenic plants provided protection against two serologically unrelated potyviruses: tobacco etch virus and potato virus Y.

However, expression of a preselected coat protein gene does not reliably confer heterologous protection to a plant. For example, transgenic squash plants containing the CMV-C coat protein gene and which have been shown to be resistant to CMV-C strain, are not protected against several highly virulent strains of CMV, including CW-V-27 and CARNA- 5. Thus, a need exists for improved methods to impart potyvirus resistance to plants.

7.2.4 USING "PATHOGEN DRIVEN RESISTANCE" (PDR) FOR DEVELOPING VIRUS RESISTANT TRANSGENIC PLANTS

Control of plant virus diseases took a major step forward in the last decade when it was shown in 1986 that the tobacco mosaic virus ("TMV") coat protein gene that was expressed in transgenic tobacco conferred resistance to TMV (Powell-Abel, P., et al., "Delay of Disease Development in Transgenic Plants that Express the Tobacco Mosaic Virus Coat Protein Gene," *Science*, 232:738-43 (1986)). The concept of pathogen-derived resistance ("PDR"), which states that pathogen genes that are expressed in transgenic plants will confer resistance to infection by the homologous or related pathogens (Sanford, J.C., et al. "The Concept of Parasite-Derived Resistance - Deriving Resistance Genes from the Parasite's Own Genome," *J. Theor. Biol.*, 113:395-405 (1985)) was introduced at about the same time. Since then, numerous reports have confirmed that PDR is a useful strategy for developing transgenic plants that are resistant to many different viruses (Lomonossoff, G.P., "Pathogen-Derived Resistance to Plant Viruses," *Ann. Rev. Phytopathol.*, 33:323-43 (1995)).

Only eight years after the report by Beachy and colleagues (Powell-Abel, P., et al., "Delay of Disease Development in Transgenic Plants that Express the Tobacco Mosaic Virus Coat Protein Gene," *Science*, 232:738-43 (1986)), Grunet, R., "Development of

Virus Resistant Plants via Genetic Engineering," Plant Breeding Reviews, 12:47-49 (1994) reviewed the PDR literature and listed the successful development of virus resistant transgenic plants to at least 11 different groups of plant viruses.

7.2.1.4.1 Utilizing The Coat Protein Genes

The vast majority of reports have utilized the coat protein genes of the viruses that are targeted for control. Although the testing of transgenic plants have been largely confined to laboratory and greenhouse experiments, a growing number of reports showed that resistance is effective under field conditions (e.g., Grumet, R., "Development of Virus Resistant Plants via Genetic Engineering," Plant Breeding Reviews, 12:47-49 (1994)). Two virus resistant crops have been deregulated by APHIS/USDA and thus are approved for unrestricted release into the environment in the U.S.A. Squash that are resistant to watermelon mosaic virus 2 and zucchini yellow mosaic potyviruses have been commercialized (Fuchs, M., et al., "Resistance of Transgenic Hybrid Squash ZW-20 Expressing the Coat Protein Genes of Zucchini Yellow Mosaic Virus and Watermelon Mosaic Virus 2 to Mixed Infections by Both Potyviruses," Bio/Technology, 13:1466-73 (1995); Tricoli, D.M., et al., "Field Evaluation of Transgenic Squash Containing Single or Multiple Virus Coat Protein Gene Constructs for Resistance to Cucumber Mosaic Virus, Watermelon Mosaic Virus 2, and Zucchini Yellow Mosaic Virus," Bio/Technology, 13:1458-65 (1995)). Also, a transgenic papaya that is resistant to papaya ringspot virus has been developed (Fitch, M. M. M., et al., "Virus Resistant Papaya Derived from Tissues Bombarded with the Coat Protein Gene of Papaya Ringspot Virus," Bio/Technology, 10:1466-72 (1992); Tennant, P.F., et al., "Differential Protection Against Papaya Ringspot Virus Isolates in Coat Protein Gene Transgenic Papaya and Classically Cross- Protected Papaya," Phytopathology, 84:1359-66 (1994)). This resistant transgenic papaya was recently deregulated by USDA/APHIS. Deregulation of the transgenic papaya is timely, because Hawaii's papaya industry is being devastated by papaya ringspot virus. Undoubtedly, more crops will be deregulated and commercialized in the near future.

7.2.1.4.2 Other effective viral genes

Interestingly, remarkable progress has been made in developing virus resistant transgenic plants despite a poor understanding of the mechanisms involved in the various forms of pathogen-derived resistance (Lomonossoff, G.P., "Pathogen-Derived Resistance to Plant Viruses," Ann.-Rev. Phytopathol., 33:323-43 (1995)). Although most reports deal

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7.1.2.1.5.1 Description (A Form of PDR)

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Plant Cell, 6:1441-53 (1994)), potexviruses (Mueller, E., et al., "Homology-Dependent Resistance: Transgenic Virus Resistance in Plants Related to Homology-Dependent Gene Silencing," Plant Journal, 7:1001-13 (1995)), and TSWV and other tospoviruses (Pang, S.Z., et al., "Resistance of Transgenic Nicotiana Benthamiana Plants to Tomato Spotted Wilt and Impatiens Necrotic Spot Tospoviruses: Evidence of Involvement of the N Protein and N Gene RNA in Resistance," Phytopathology, 84:243-49 (1994); Pang, S.-Z., et al., "Different Mechanisms Protect Transgenic Tobacco Against Tomato Spotted Wilt Virus and Impatiens Necrotic Spot Tospoviruses," Bio/Technology 11:819-24 (1993)). More recent work has shown that RNA-mediated resistance also occurs with the comovirus cowpea mosaic virus (Sijen, T., et al., "RNA-Mediated Virus Resistance: Role of Repeated Transgene and Delineation of Targeted Regions," Plant Cell, 8:2227-94 (1996)) and squash mosaic virus (Jan, F.-J., et al., "Genetic and Molecular Analysis of Squash Plants Transformed with Coat Protein Genes of Squash Mosaic Virus," Phytopathology, 86:S16-17 (1996)).

7.2.1.5.2 The Mechanism(S) of RNA-Mediated Resistance

Major advances towards understanding the mechanism(s) of RNA-mediated resistance were made by Dougherty and colleagues in a series of experiments with TEV and PVY. Using TEV, Lindbo, J.A., "Pathogen-Derived Resistance to a Potyvirus Immune and Resistant Phenotypes in Transgenic Tobacco Expressing Altered Forms of a Potyvirus Coat Protein Nucleotide Sequence," Mol. Plant Microbe Interact., 5:144-53 (1992) and Lindbo, J.A., et al., "Untranslatable Transcripts of the Tobacco Etch Virus Coat Protein Gene Sequence can Interfere with Tobacco Etch Virus Replication in Transgenic Plants and Protoplasts," Virology, 189:725-33 (1992) showed that transgenic plants expressing translatable full length coat protein, truncated translatable coat protein, antisense coat protein genes, and nontranslatable coat protein gene had various phenotypic reactions after inoculation with TEV. Transgenic plants displayed resistance, recovery (inoculated plants initially show systemic infection but younger leaves that develop later are symptomless and resistant to the virus), or susceptible phenotypes. Furthermore, they showed that leaves of resistant plants and asymptomatic leaves of recovered plants had relatively low levels of steady state RNA when compared to those in leaves of susceptible plants (Lindbo, J.A., et al., "Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resistance," Plant Cell, 5:1749-

59 (1993)). However, nuclear run off experiments showed that those plants with low levels of steady state RNA had higher transcription rates of the viral transgene than those plants that were susceptible (and had high steady state RNA levels). To account for these observations, it was proposed "that the resistant state and reduced steady state levels of transgene transcript accumulation are mediated at the cellular level by a cytoplasmic activity that targets specific RNA sequences for inactivation," (Lindbo, J.A., et al., "Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resistance," Plant Cell, 5:1749-59 (1993)). It was also suggested that the low steady state RNA levels may be due to post-transcriptional gene silencing, a phenomenon that was first proposed by de Carvalho, F., et al., "Suppression of beta-1,3-glucanase Transgene Expression in Homozygous Plants," EMBO J., 11:2595-602 (1992) for the suppression of P-1,3-glucanase transgene in homozygous transgenic plants.

An RNA threshold model was proposed to account for the observations (Lindbo, J.A., et al., "Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resistance," Plant Cell, 5:1749-59 (1993)). Basically, the model states that there is a cytoplasmic cellular degradation mechanism that acts to limit the RNA levels in plant cells, and that this mechanism is activated when the transgenic RNA transcript goes above a threshold level. The degradation mechanism is specific for the transcript that goes above the threshold level; and if the transcripts that go above a certain threshold is a viral transgene, the virus resistance state is observed in the plant, because the degradation mechanism also targets, for inactivation, the specific sequences of the incoming virus. The model also accounts for the 'recovery' of transgenic plants by suggesting that viral RNA from the systemically invading virus triggers the phenomenon in some transgenic plants that have two copies of the transgenes. Plants that had more than three copies of the transgenes caused the threshold level to be surpassed without the invasion of virus (Goodwin, J., et al., "Genetic and Biochemical Dissection of Transgenic RNA-Mediated Virus Resistance," Plant Cell, 8:95-105 (1996); Smith, H.A., et al., "Transgenic Plant Virus Resistance Mediated by Untranslatable Sense RNAs: Expression, Regulation, and Fate of Nonessential RNAs," Plant Cell, 6:1441-53 (1994)). Although the degradation mechanism is not clear, it is proposed that a cellular RNA dependent RNA polymerase ("RdRp") binds to the transcript

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cauliflower mosaic virus (CaMV) 35S promoter, modified to contain two tet operons and linked to the chloramphenicol acetyltransferase (cat) gene (Gatz and Quail, 1988), or modified to contain three tet operons and linked to the beta-glucuronidase (gus) gene (Gatz, et al., 1992). So long as the Tn10 tet repressor gene is active, the modified promoter is repressed by the interaction of the repressor with the tet operons, and the cat or gus gene is not expressed. The presence of tetracycline inhibits repressor binding, enabling expression of the cat or gus gene.

7.2.3 RECOMBINANT PRODUCTION OF PROTEINS

A major commercial focus of biotechnology is the recombinant production of proteins, including both industrial enzymes and proteins that have important therapeutic uses.

7.2.3.1 Recombinant proteins produced in microbial hosts

Therapeutic proteins are commonly produced recombinantly by microbial expression systems, such as in *E. coli* and the yeast system *S. cerevisiae*. To date, the cost of recombinant proteins produced in a microbial host has limited the availability of a variety of therapeutically important proteins, such as human serum albumin (HSA) and α_1 -antitrypsin (AAT), to the extent that the proteins are in short supply.

7.2.31.1 Inability of microbial systems to glycosylate (properly) mammalian proteins

Some therapeutic proteins appear to rely on glycosylation for optimal activity or stability, and the general inability of microbial systems to glycosylate or properly glycosylate mammalian proteins has also limited the usefulness of these recombinant expression systems. In some cases, proper protein folding cannot take place, because of the need for mammalian-specific foldases or other folding conditions.

7.2.3.1.2 Cost per weight ratio and other problems of mammalian expression systems

To some extent, protein expression in cultured mammalian cells, or in transgenic animals may overcome the limitations of microbial expression systems. However, the cost per weight ratio of the protein is still high in mammalian expression systems, and the risk of protein contamination by mammalian viruses may be a significant regulatory problem. Protein production by transgenic animals also carries the risk of genetic variation from one generation to another. The attendant risk is variation in the recombinant protein produced, for example, variation in protein processing to yield a native active protein with different N-terminal residue.

7.2.3.2 Alternative protein expression system to overcome problems of microbial and mammalian systems

It would therefore be desirable to produce selected therapeutic and industrial proteins in a protein expression system that largely overcomes problems associated with

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7.2.3.2.3 Use: Human Serum Albumin

Human serum albumin (HSA) is expressed as a 609 amino acid prepro- protein which is further processed by removal of an amino-terminal peptide and an additional six amino acid residues to form the mature protein. The mature protein found in human serum is a monomeric, unglycosylated protein 585 amino acids in length (66 kDal), with a globular structure maintained by 17 disulfide bonds. The pattern of disulfide links forms a structural unit of one small and two large disulfide-linked double loops (Geisow, M.J. et al. (1977) *Biochem. J.* 163:477-484) which forms a high-affinity bilirubin binding site.

Because of the key role of HSA as an osmotic stabilizing agent, and because of its broad clinical potential in, e.g., plasma replacement therapy, there has been an active interest in

1. **Содержание:** 1. Введение. 2. Описание системы. 3. Требования к системе. 4. Структура системы. 5. Описание компонентов. 6. Описание процессов. 7. Описание данных. 8. Описание интерфейсов. 9. Описание тестирования. 10. Заключение.

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7.2.4 PRACTICAL METHOD FOR THE COMMERCIAL PRODUCTION OF TRANSGENIC PLANTS

Translating genetic engineering theory into practice, however, and then furthermore into a commercially practical reality, requires ingenuity. Gene transplantation in plants has already been accomplished at this writing--and examples are cited below--but heretofore no practical method for the commercial production of transgenic plants has been perfected.

7.2.4.1 Tissue culture propagation

Apart from the transgenic plant technology per se, it is known to propagate plants by replicating plant cells in culture, or "tissue culture." An early motivating force in the development of tissue culture was the desire to improve upon the relatively slow and low yields of vegetative propagation with the quick and exponential proliferation of new plants from cell culture. Tissue culture methods are made possible by the plant physiological phenomenon of callus formation. When a plant is wounded, a patch of soft cells called a calli grows over the wound and, with time, phenolic compounds accumulate in the soft cells and harden, effectively sealing the wound. While hardened callus is the plant equivalent of scar tissue, callus is different from mammalian scar tissue with respect to its regenerative properties. If a piece of young, still-soft callus is removed and placed in a culture medium containing salts, sugars, vitamins, amino acids and the appropriate plant growth hormones, rather than harden, the cells will continue to divide and give rise to a disorganized mass of undifferentiated cells called a "callus culture." Plant or seedling "explants," or tissue samples, will likewise grow into similar cell cultures. The cultured cells can further be induced to redifferentiate into shoots, roots or whole plants by further culturing with the necessary hormones and growth media.

One of the most serious drawbacks with tissue culture propagation techniques has been the morphologic variation from generation to generation, a problem which is particularly notable in certain species and varieties. For example, as reported in Cassells, A.C., and Carney, B.F., "Adventitious regeneration in Pelargonium x domesticum Bailey," Acta Horticulturae, 212(11), 419-425 (1987), in stem and petiole tissue cultures of Grand Slam (as an example of P. domesticum, also known as Regal Pelargoniums or "Martha Washington" geraniums), up to 16% of the adventitious regenerants were

variants, depending on the explant origin. The authors concluded that genome instability in Grand Slam and presumably other P. domesticum varieties may produce useful variation but mitigates against the use of adventitious regeneration in micropropagation.

The findings of Cassells et al. are consistent with the earlier work of Skirvin, R.M. and Janick, Jules, "Tissue Culture-Induced variation in Scented Pelargonium ssp.," Amer. Soc. Hort. Sci. , 101 (3) , 281-290 (1976). Skirvin et al. compared tissue culture propagated Pelargonium plants (from root cuttings, petiole cuttings or calliclones) with plants derived from vegetative propagation, i.e., stem cuttings. The plants derived from stem cuttings were all uniform and identical to the parental clone, whereas those from the root cuttings, petiole cuttings or calliclones were all morphologically distinct with the degree of variability depending upon the cultivar. The authors conclude that the variability associated with calliclones derived from tissue culture is a pool on which selection can be imposed, implying conversely that tissue culturing of this type is inappropriate for use in attempting reliable regeneration of Pelargonium x domesticum varieties.

Other varieties and species, besides Pelargonium x domesticum, are known and/or believed to suffer morphologic variation when propagated using tissue culture. It can be easily appreciated that any substantial morphologic variation in propagation is unacceptable for commercial propagation of a desired variety or species. Thus, tissue culture methods are not always acceptable for commercial use, even with the potentially much larger yields achievable as compared with prior art vegetative propagation techniques.

7.2.4.2 Gene transplantation

Apart from tissue culture considerations, gene transplantation in plants has achieved some success at this writing. Gene introduction is generally accomplished with a vector such as Agrobacterium. As this technology developed, it was noted that Crown Gall tumors of plants arose at the site of infection of some species of the bacterium Agrobacterium. The cells of Crown Galls acquire the properties of independent, unregulated growth. In culture, such transformed cells grow in the absence of the plant hormones usually necessary for plant cell growth, and the cells retain the transformed phenotype even in the absence of the bacterium. The tumor-inducing agent in

Agrobacterium is a plasmid that integrates some of its DNA into the chromosome of the host plant cells. Ti (tumor-inducing) plasmids exist in Agrobacterium cells as independently replicating genetic units.

Ti plasmids are maintained in Agrobacterium because part of the plasmid DNA, the T-DNA, carries the genes coding for the synthesis of amino acids called opines. The infected plant cell is induced to synthesize these amino acids, but the plant cannot use these amino acids. The Ti plasmid is believed to carry genes coding for enzymes that can degrade opines. Thus, Ti plasmids both make and degrade opines, within the plant cell, which the plant cell cannot metabolically use--presumably giving a selective advantage to the Agrobacterium at least with respect to utilization of the opine metabolites. A second set of genes in T-DNA codes for enzymes which lead to production of hormones which, in turn, cause the infected plant cell to divide in an unregulated way.

In summary terms, T-DNA enters a plant cell by what amounts to the equivalent of bacterial conjugation between the Agrobacterium and the plant cell. In other words, an Agrobacterium organism and a plant cell transfer their DNA in a process analogous to mating. Ultimately, T- DNA becomes incorporated into the genomic plant cell DNA in the plant cell nucleus.

All of the above background illustrates how Agrobacterium species can serve well as vectors for genetic transformation of plant cells. Early gene transfer using Ti plasmids, T-DNA and Agrobacterium was accomplished by the cointegration method, in which T-DNA was first cloned into a standard E. coli cloning vector, and the plant gene was subsequently cloned into a second cloning site carried by the vector. This intermediate vector was introduced into Agrobacterium organisms containing intact Ti plasmids.

Recombination occurred between the homologous regions of the intermediate vector and the wild-type Ti plasmid, and on infection of a plant with the Agrobacterium the recombinant plasmid is transferred to the plant cells.

7.2.4.3 The binary system

plasmid which can be used to transfer to the plant genome the genes necessary for improved root growth in culture. The use of sterilized petioles as the source of explant material for plant transformation and culture is disclosed.

U.S. Patent No. 5,276,268 to Strauch et al., entitled "Phosphinothricin-Resistance Gene, and Its Use," is directed to the transfer of phosphinothricin-resistance gene into plants using Agrobacterium species. A modification of the binary vector method is discussed, and the phosphinothricin-resistance gene nucleic acid sequences are provided.

U.S. Patent No. 5,283,184 to Jorgenson et al. is entitled "Genetic Engineering of Novel Plant Phenotypes" and discusses transgenote formation and propagation in tissue culture, as well mentioning Pelarcioniums and geraniums (and many other plants) by name. The tissue culture propagation of morphologically conserved transgenotes is not discussed.

U.S. Patent No. 5,286,635 to Hanson et al., entitled "Genetically, Transformed Pea Plants and Methods for Their Production," discloses the transfer of desired gene sequences into pea plants by incubating pea plant explants (preferably not callus) with Agrobacterium vectors containing the desired gene sequence. Mature seed material is used as the explant source. The issue of total morphologic conservation is not addressed.

7.2.4.4 A method for commercially viable production of transgenic plants in which plants undergo minimal, and thus commercially acceptable, morphologic variation as a result of tissue culture propagation

Thus while certain inroads have been made in the area of tissue culture plant propagation as well as in plant gene transfer, a need remains for a method for the commercially viable production of transgenic plants in which the plants undergo only minimal, and thus commercially acceptable, morphologic variation as a result of tissue culture propagation.

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cloning those individual loci which contribute predominantly to the expression of the trait. Determination of genotypic information from phenotypic values is further imprecise because evaluation of the trait may frequently be confounded by environmental effects (Berger, "Multiple-Trait Selection Experiments: Current Status, Problem Areas and Experimental Approaches." In: Proceedings of the International Conference on Quantitative Genetics (Pollack et al., Eds.), p. 191-204 (Iowa St. Press 1977)).

Clearly, one area in which biotechnology may have a significant impact on plant improvement is in the development of new methods to identify and characterize the role of individual plant genes in quantitative trait expression. Following the development of a new class of plant molecular markers based on restriction fragment length polymorphisms, termed "RFLPs", (Helentjaris et al., Plant Mol. Bio. 5:109-118 (1985)) ("Helentjaris et al. I"), the processes to identify such loci and discriminate gene effects have been invented and are described and claimed herein. This and all other publications noted herein are hereby incorporated by reference. This will undoubtedly benefit plant improvement, not only within the context of conventional breeding approaches, but also by providing a means for identifying appropriate loci for future cloning and direct gene transfer efforts.

addition of polyadenylated ribonucleotides to the 3' end of the transcribed mRNA sequences. Preferably, the virus is a plant-associated virus, such as a potyvirus.

Thus, the present DNA molecule can be employed as a chimeric recombinant "expression construct," or "expression cassette" to prepare transgenic plants that exhibit increased resistance to infection by at least two plant viruses, such as potyviruses. The present cassettes also preferably comprise at least one selectable marker gene or reporter gene which is stably integrated into the genome of the transformed plant cells in association with the viral genes. The selectable marker and/or reporter genes facilitate identification of transformed plant cells and plants. Preferably, the virus gene array is flanked by two or more selectable marker genes, reporter genes or a combination thereof.

Another aspect of the present invention is a method of preparing a virus-resistant plant, such as a dicot, comprising:

(a) transforming plant cells with a chimeric recombinant DNA molecule comprising a plurality of DNA sequences, each comprising a promoter functional in said plant cells, operably linked to a DNA sequence, which encodes a protein associated with a virus which is capable of infecting said plant; (b) regenerating said plant cells to provide a differentiated plant; and (c) identifying a transformed plant which expresses the DNA sequences so as to render the plant resistant to infection by said viruses, preferably at substantially equal levels of resistance to infection by each virus.

Yet another object of the present invention is to provide a method for providing resistance to infection by viruses in a susceptible Cucurbitaceae plant which comprises:

(a) transforming Cucurbitaceae plant cells with a DNA molecule encoding a plurality of proteins from viruses which are capable of infecting said Cucurbitaceae plant; (b) regenerating said plant cells to provide a differentiated plant; and (c) selecting a transformed Cucurbitaceae which expresses the virus proteins at levels sufficient to render the plant resistant to infection by said viruses.

It is a further object of the present invention to provide multi-virus resistant transformed plant which contains stably-integrated DNA sequences encoding virus proteins.

7.3.3 CONTROLLING GENE EXPRESSION WITH EXTERNAL STIMULUS

The present invention involves, in one embodiment, the creation of a transgenic plant that contains a gene whose expression can be controlled by application of an external stimulus. This system achieves a positive control of gene expression by an external stimulus, without the need for continued application of the external stimulus to maintain gene expression. The present invention also involves, in a second embodiment, the creation of transgenic parental plants that are hybridized to produce a progeny plant expressing a gene not expressed in either parent. By controlling the expression of genes that affect the plant phenotype, it is possible to grow plants under one set of conditions or in one environment where one phenotype is advantageous, then either move the plant or plant its seed under another set of conditions or in another environment where a different phenotype is advantageous. This technique has particular utility in agricultural and horticultural applications.

In accordance with one embodiment of the invention, a series of sequences is introduced into a plant that includes a transiently-active promoter linked to a structural gene, the promoter and structural gene being separated by a blocking sequence that is in turn bounded on either side by specific excision sequences, a repressible promoter operably linked to a gene encoding a site-specific recombinase capable of recognizing the specific excision sequences, and a gene encoding a repressor specific for the repressible promoter whose function is sensitive to an external stimulus. Without application of the external stimulus, the structural gene is not expressed. Upon application of the stimulus, repressor function is inhibited, the recombinase is expressed and effects the removal of the blocking sequence at the specific excision sequences, thereby directly linking the structural gene and the transiently-active promoter.

In a modification of this embodiment, the sequences encoding the recombinase can be introduced separately into the plant via a viral vector.

In an alternative embodiment, no repressor gene or repressible promoter is used. Instead, the recombinase gene is linked to a germination-specific promoter and introduced into a separate plant from the other sequences. The plant containing the transiently-active promoter, blocking sequence, and structural gene is then hybridized with the plant containing the recombinase gene, producing progeny that contain all of the sequences.

When the second transiently-active promotor becomes active, the recombinase removes the blocking sequence in the progeny, allowing expression of the structural gene in the progeny, whereas it was not expressed in either parent.

In still another embodiment, the recombinase gene is simply linked to an inducible promoter. Exposure of the plant to the induce specific for the inducible promoter leads to the expression of the recombinase gene and the excision of the blocking sequence.

In all of these embodiments, the structural gene is expressed when the transiently-active promoter becomes active in the normal course of growth and development, and will continue to be expressed so long as the transiently-active promoter is active, without the necessity of continuous external stimulation. This system is particularly useful for developing seed, where a particular trait is only desired during the first generation of plants grown from that seed, or a trait is desired only in subsequent generations.

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7.3.4 PREPARING PLANTS WHICH ARE RESISTANT TO MULTIPLE VIRUSES

It is still a further object of the present invention to provide virus resistant transformed plant cells which contain a plurality of viral genes, i.e., 2-7 or more genes, which are expressed as virus proteins, such as coat proteins, proteases and/or replicases, from the same virus strain, from different virus strains as from different members of the virus group, such as the potyvirus group. Representative viruses from which these DNA sequences can be isolated include, but are not limited to, potato virus X (PVX), potyviruses such as potato virus Y (PVY), cucomovirus (CMV), tobacco vein mottling virus, watermelon mosaic virus (WMV), zucchini yellow mosaic virus (ZYMV), bean common mosaic virus, bean yellow mosaic virus, soybean mosaic virus, peanut mottle virus, beet mosaic virus, wheat streak mosaic virus, maize dwarf mosaic virus, sorghum mosaic virus, sugarcane mosaic virus, johnsongrass mosaic virus, plum pox virus, tobacco etch virus, sweet potato feathery mottle virus, yam mosaic virus, and papaya ringspot virus (PRV), cucomoviruses, including CMA and comovirus.

Generally, a potyvirus is a single-stranded RNA virus that is surrounded by a repeating proteinaceous monomer, which is termed the coat protein (CP). The encapsidated virus has a flexous rod morphology. The majority of the potyviruses are transmitted in a nonpersistent manner by aphids. As can be seen from the wide range of crops affected by potyviruses, the host range includes such diverse families of plants, but is not limited to Solanaceae, Chenopodiaceae, Gramineae, Compositae, Leguminosae, Dioscoreaceae, Cucurbitaceae, and Caricaceae.

The present invention is particularly directed to preparing plants which are resistant to multiple viruses. It is well known that particular plant types are often susceptible to more than one virus. Although PDR is an excellent approach to controlling the damaging effects of plant viruses, incorporating multiple virus resistance in a given plant can be challenging. For example, identifying and producing full length viral genes to transform plants can be expensive and time consuming. Further, such genes may be so large that they need to be incorporated in different expression systems which must be separately incorporated in plants.



7.3.4.1 Using short fragments of viral genes to impart resistance

Rather than attempting to incorporate full length viral genes in a plant, the present invention uses short fragments of such genes to impart resistance to the plant against a plurality of viral pathogens. These short fragments, which each by themselves have insufficient length to impart such resistance, are more easily and cost effectively produced than full length genes. There is no need to include in the plant separate promoters for each of the fragments; only a single promoter is required. Moreover, such viral gene fragments can preferably be incorporated in a single expression system to produce transgenic plants with a single transformation event.

7.3.4.1.1 Example: papaya ringspot virus

The impact of this simple strategy for multiple virus resistant transgenic plants could have far reaching effects in agriculture. An example is the case of papaya ringspot virus ("PRV"). Transgenic papaya with the coat protein gene of the PRV strain from Hawaii have been developed and found to be highly resistant under greenhouse and long-term field conditions. However, that papaya is largely susceptible to strains from other parts of the world, including Jamaica, Thailand, and Brazil.

Apparently, PRV resistance in papaya is highly specific and a number of transgenic papaya lines will need to be developed with different coat protein genes of the target countries to control the virus worldwide. With the present invention, a transgenic papaya could be developed with resistance to all PRV strains using viral gene fragments that total less than 1,000 base pairs plus a silencer DNA of about 400 bp; by comparison, the PRV coat protein gene alone is about 1,000 bp.

7.3.4.1.2 Example: potato leaf roll virus, potato virus Y, and potato virus X

Another use of the present invention involves imparting resistance against a plurality of different viruses. For example, in potato, the present invention can be employed to impart resistance against potato leaf roll virus, potato virus Y, and potato virus X. To effect such resistance, in accordance with the present invention, a DNA construct, driven by a single promoter, and containing a portion of the potato leafroll virus replicase encoding gene, a portion of the potato virus Y coat protein encoding gene, and a portion of the gene encoding the movement protein of potato virus X can be produced and

transformed into potato. As a result, transgenic potato with resistance to potato leafroll virus, potato virus Y, and potato virus X can be produced by a single transformation event. This constitutes a significant advance beyond incorporating full length versions of each of the genes with separate promoters together in a single expression vector or in separate vectors.

7.3.4.1.3 Example: multiple resistance

Another use of the present invention involves imparting resistance to cucurbits against a number of viruses. For example, in squash, the present invention can be utilized to impart multiple resistance to zucchini yellow mosaic virus, papaya ringspot virus, watermelon mosaic virus IT, and squash mosaic virus. For example, a construct containing a portion of the coat protein encoding gene or a portion of the replicase encoding gene from each of these viruses, driven by a single promoter, can be produced and transformed into squash. The resulting transgenic squash is resistant to all of these viruses.

The present invention is exemplified primarily by the insertion of multiple virus cp expression cassettes into a binary plasmid and subsequent characterization of resulting plasmids. Combinations of CMV, ZYMV, WMV-2, SQMV, and PRV coat protein expression cassettes were placed in the binary plasmid pPRBN. Subsequently, binary plasmids harboring multiple cp expression cassettes were mobilized into Agrobacterium for use in plant transformation procedures. Binary plasmids harboring multiple expression cassettes are employed to transfer two or more virus coat protein transformation-susceptible genes into plants, such as members of the Cucurbitaceae family, along with the associated selectable marker and/or reporter genes.

7.3.5 IMPARTING OTHER TRAITS TO PLANTS

In addition to conferring on plants resistance to multiple viral diseases, the present invention can be utilized to impart other traits to plants. It is often desirable to incorporate a number of traits to a transgenic plant besides disease resistance. For example, color, enzyme production, etc. may be desirable traits to confer on a plant. However, transforming plants with a plurality of such traits encounter the same difficulties discussed above with respect to disease resistance. The present invention may be likewise useful in alleviating these problems with respect to traits other than disease resistance.

Thus, the present invention provides a genetic engineering methodology by which multiple traits can be manipulated and tracked as a single gene insert, i.e., as a construct which acts as a single gene which segregates as a single Mendelian locus. Although the invention is exemplified via virus resistance genes, in practice, any combination of genes could be linked. Therefore one could track a block of genes that provide traits such as disease resistance, plus enhanced herbicide resistance, plus extended shelf life, and the like, by simply tracking the linked selectable marker or reporter gene which has been incorporated into the transformation vector.

It was also discovered that when multiple tandem genes are inserted, they preferably all exhibit substantially the same degrees of efficacy, and more preferably substantially equal degrees of efficacy, wherein the term "substantial" as it relates to viral resistance is defined with reference to the assays described in the examples hereinbelow. For example, if one examines numerous transgenic lines containing an intact ZYMV and WMV-2 coat protein insert, one finds that if a line is immune to infection by ZYMV it is also immune to infection by WMV-2. Similarly, if a line exhibits a delay in symptom development to ZYMV it will also exhibit a delay in symptom development to WMV2. Finally, if a line is susceptible to ZYMV it will be susceptible to WMV-2. This phenomenon is unexpected. If there were not a correlation between the efficacy of each gene in these multiple gene constructs this approach as a tool in plant breeding would probably be prohibitively difficult to use. Even with single gene constructs, one must test numerous transgenic plant lines to find one that displays the appropriate level of efficacy. The probability of finding a line with useful levels of expression can range from 10-50% (depending on the species involved).

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If the efficacy of individual genes in a Ti plasmid containing multiple genes were independent, the probability of finding a transgenic line that was resistant to each targeted virus would decrease dramatically. For example, in a species in which there is a 10% probability of identifying a line with resistance using a single gene insert, is transformed with a triple-gene construct CZW and each gene display an independent levels of efficacy, the probability of finding a line with resistance to CMV, ZYMV and WMV-2 would be $0.1 \times 0.1 \times 0.1 = 0.001$ or 0.1 %. However, since the efficacy of multivalent genes is not independent of each other the probability of finding a line with resistance to CMV, ZYMV and WMV-2 is still 10% rather than 0.1 %. Obviously this advantage becomes more pronounced as constructs containing four or more genes are used.

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In order to meet this need, the present method is a process for commercially propagating plants by tissue culture in such a way as both to conserve desired plant morphology and to transform the plant with respect to one or more desired genes. The method includes the steps of (a) creating an Agrobacterium vector containing the gene sequence desired to be transferred to the propagated plant, preferably together with a marker gene; (b) taking one or more petiole explants from a mother plant and inoculating them with the Agrobacterium vector; (c) conducting callus formation in the petiole sections in culture, in the dark; and (d) culturing the resulting callus in growth medium having a benzylamino growth regulator such as benzylaminopurine or, most preferably, benzylaminopurine-riboside. Additional optional growth regulators including auxins and cytokinins (indole butyric acid, benzylamine, benzyladenine, benzylaminopurine, alpha naphthylacetic acid and others known in the art) may also be present.

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The method includes obtaining monocot cells transformed with a chimeric gene having (i) a monocot transcriptional regulatory region, inducible by addition or removal of a small molecule, or during seed maturation, (ii) a first DNA sequence encoding the heterologous protein, and (iii) a second DNA sequence encoding a signal peptide. The second DNA sequence is operably linked to the transcriptional regulatory region and to the first DNA sequence. The first DNA sequence is in translation-frame with the second DNA sequence, and the two sequences encode a fusion protein.

In one embodiment of the method, the first DNA sequence encodes pro-subtilisin BPN' (proBPN'), the cultivating includes cultivating the transformed cells at a pH between 5 and 6, and the isolating step includes incubating the proBPN' to under condition effective to allow its autoconversion to active mature BPN'. In another embodiment, the first DNA sequence encodes mature BPN', and the cells are transformed with a second chimeric gene containing (i) a transcriptional regulatory region inducible by addition or

removal of a small molecule, (ii) a third DNA sequence encoding the pro-peptide moiety of BPN', and (iii) a fourth DNA sequence encoding a signal polypeptide. The fourth DNA sequence is operably linked to the transcriptional regulatory region and to the third DNA sequence, and the signal polypeptide is in translation-frame with the pro-peptide moiety and is effective to facilitate secretion of expressed pro-peptide moiety from the transformed cells. The cultivating step includes cultivating the transformed cells at a pH between 5 and 6, and the isolating step includes incubating the mature BPN and the pro-moiety under conditions effective to allow the conversion of BPN' by the pro-moiety to active mature BPN'.

7.3.8.1 Inducing the transcriptional regulatory region

In other embodiments of the method, the transcriptional regulatory region may be a promoter derived from a rice or barley α -amylase gene, including RAmylA, RAmylB, RAmy2A, RAmy3A, RAmy3B, RAmy3C, RAmy3D, RAmy3E, pM/C, gKAmyl4l, gKAmyl55, Amy32b, or HV18. The chimeric gene may further include, between the transcriptional regulatory region and the fusion protein coding sequence, the 5' untranslated region (5' UTR) of an inducible monocot gene such as one of the rice or barley α -amylase genes described above. One preferred 5' UTR is that from the RAmylA gene, which is effective to enhance the stability of the gene transcript. The chimeric gene may further include, downstream of the coding sequence, the 3' untranslated region (3' UTR) from an inducible monocot gene, such as one of the rice or barley α -amylase genes mentioned above. One preferred 3'UTR is from the RAmylA gene.

7.3.8.2 Preferred Promoters

Where the method is employed in protein production in a monocot cell culture, preferred promoters are the RAmy3D and RAmy3E gene promoters, which are upregulated by sugar depletion in cell culture. Where the gene is employed in protein production in germinating seeds, a preferred promoter is the RAmylA gene promoter, which is upregulated by gibberellic acid during seed germination. Where gene is upregulated during seed maturation, a preferred promoter is the barley endosperm-specific B1-hordein promoter.

7.3.8.3 Product: A Mature Heterologous Protein

Questions and answers on the new *Journal of the History of Biology* are available at <http://www.jhb.oxfordjournals.org>

DOUGLAS J. HARRIS, JR. is a senior research advisor at the U.S. Environmental Protection Agency, Washington, D.C.

These and other objects and features of the invention will be more fully understood when the following detailed description of the invention is read in conjunction with the accompanying drawings.

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RFLPs are differences observed between genotypes in the fragment lengths of restriction endonuclease-digested DNA. RFLPs occur as a result of base pair or positional changes in the restriction enzyme recognition sites which flank a chromosomal location and can be detected by hybridization of labelled DNA clones containing sequences that are homologous to a portion of the chromosomal fragment. Hybridization with a unique cloned sequence can permit the identification of a specific chromosomal region (locus).

This technology employs cloned DNA fragments to detect differences between individuals at the DNA sequence level. When genomic DNAs from two genetically distinct individuals are digested with a restriction enzyme, electrophoresed and probed with a labelled DNA clone, polymorphisms in the hybridization patterns sometimes result due to sequence differences between the individuals. The term "restriction fragment length polymorphism" has been coined to describe this variation.

Differences in fragment lengths which are revealed, for example, by agarose gel electrophoresis, function as alleles of that RFLP. Thus, RFLPs can serve as genetic markers in a manner analogous to conventional morphological or isozyme markers. Unlike most genetic markers, however, they are not the products of transcription and translation. Additionally, RFLP possess certain additional advantages over previously available genetic markers. First, RFLPs reflect existing differences between genetically distinct individuals. The potential number of RFLPs for all practical purposes is thus unlimited, as digestion of the genomic DNA of any higher eukaryote with a six base recognition enzyme will generate more than a million fragments, many of which can be polymorphic.

(1987)). The level of informativeness of these RFLPs is great. In a study involving the maize cross Tx303X Co159, only 13 informative isozyme loci with very biased coverage of less than the 10 chromosomes were available. In contrast, using RFLP analysis, more than 99 informative RFLPs covering all 10 chromosomes can be analyzed in the same cross (unpublished data). In a recent comparative survey of Corn Belt germplasm RFLPs averaged greater than five alleles per locus while isozyme loci averaged less than two (M. Walton and T. Helentjaris, abstract).

7.3.9.3 RFLP Markers in Plant Breeding

RFLP markers rarely possess detectable phenotype effects of their own, so they can be utilized in economic lines without detriment and many can be evaluated at one time without the pleiotropic effects often seen with phenotypic markers. Evaluation can be performed on small amounts of DNA obtained from plant tissue at virtually any stage of plant development from roots, to shoots, to fruits, or even with tissue culture material. Evaluation of RFLPs is not affected by environmental factors and greenhouse-grown plants will not differ from field-grown plants when tested. Finally, the evaluation of RFLPs reveals the exact genotype, so the heterozygous state can be differentiated from the homozygous condition at any chromosomal location.

Many of the potential applications and theoretical advantages of RFLPs compared to more conventional phenotypic or isozyme marking systems have been described previously. Helentjaris et al. I. In one application of the use of RFLP markers in plant studies, genetic linkage maps based on these markers have been constructed for both maize and tomato (Helentjaris et al., Theor. Appl. Genet. 72:761-769 (1986)) ("Helentjaris et al. II"). Similar linkage maps are also being constructed for other crop species, such as Brassica Figdore et al., Theor. Appl. Genetics. 75:833-840 (1988); Slocum et al., In "Genetic Maps" (S. J. O'Brien, ed.), 5th Edition, Cold Spring Harbor Press, N.Y. (1990)). Close to 120 RFLPs in tomato have been arranged into linkage groups by comparing segregation patterns in an F₂ population derived from homozygous parental lines. Approximately 70 RFLPs have been mapped by another group of workers. Bernatzky and Tanksley, Genetics 112:887- 898 (1986). Over 300 RFLPs in maize have been arranged into linkage groups (Helentjaris et al., 1986; Helentjaris et al., 1987). The locations of the maize RFLP loci have been correlated to the conventional maize genetic map by analyzing

the inheritance patterns of the RFLPs in maize lines monosomic for different chromosomes (Helentjaris et al., Proc. Nat. Acad. Sci. USA 83:6035-6039 (1986)) ("Helentjaris et al. III"), by establishing linkage relationships with isozyme markers, cloned genes, and morphological markers with previously identified chromosomal locations (Wright et al., MNL 61:89-90 (1987)), and by analyzing inheritance patterns in B-A translocation stocks (Helentjaris et al., Weber and Helentjaris, Genetics 121:583-590 (1989). The resulting map shows the resolution possible.

Numerous direct applications of RFLP technology to facilitate plant breeding programs have been suggested. Helentjaris et al. I. Because of the large numbers of RFLP markers available in a population of interest, one of the more important applications of RFLPs may be as markers linked to genes affecting the expression of quantitatively inherited traits. In this application, RFLPs function as indirect selection criteria for traits which are difficult or expensive to evaluate phenotypically. A prerequisite for the use of RFLPs as indirect selection criteria is the identification of RFLPs closely linked to the quantitative trait loci (QTL) affecting expression of the trait of interest.

Currently, the introgression of quantitative traits from one germplasm to another involves the identification of favorable genotypes in segregating generations followed by repeated backcrossing to commercially acceptable cultivars. This procedure is feasible for simply inherited quantitative traits, but as the number of genes controlling a trait increases, screening the number of F₂ segregants required to identify at least one individual which represents the ideal (homozygous) genotype quickly becomes prohibitive. For example, with one gene and two alleles of equal frequency, the probability of recovering a desirable genotype in the F₂ generation is 1/4. However, if the number of genes is increased to 5 or 10, the probability of recovering an ideal genotype in the F₂ population is reduced to approximately one in one thousand and one in one million, respectively. Thus, to identify desirable segregants, one must either reduce the number of segregants needed or have available very efficient screening procedures. Additionally, in situations where environmental effects interfere with the ability to draw accurate genotypic information from the phenotype, large allocations of time and resources are required to evaluate progeny in replicated trials within several target environments.

PARTICULAR DEFINITIONS

The terms below have the following meaning, unless indicated otherwise in the specification.

As used in this specification, a "transiently-active promoter" is any promoter that is active either during a particular phase of plant development or under particular environmental conditions, and is essentially inactive at other times.

A "plant active promoter" is any promoter that is active in cells of a plant of interest. Plant-active promoters can be of viral, bacterial, fungal, animal or plant origin.

A gene that results in an altered plant phenotype is any gene whose expression leads to the plant exhibiting a trait or traits that would distinguish it from a plant of the same species not expressing the gene. Examples of such altered phenotypes include a different growth habit, altered flower or fruit color or quality, premature or late flowering, increased or decreased yield, sterility, mortality, disease susceptibility, altered production of secondary metabolites, or an altered crop quality such as taste or appearance.

A gene and a promoter are to be considered to be operably linked if they are on the same strand of DNA, in the same orientation, and are located relative to one another such that the promoter directs transcription of the gene (i.e. in cis). The presence of intervening DNA sequences between the promoter and the gene does not preclude an operable relationship.

A "blocking sequence" is a DNA sequence of any length that blocks a promoter from effecting expression of a targeted gene.

A "specific excision sequence" is a DNA sequence that is recognized by a site-specific recombinase.

A "recombinase" is an enzyme that recognizes a specific excision sequence or set of specific excision sequences and effects the removal of, or otherwise alters, DNA between specific excision sequences.

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include sugars, sugar-derivatives (including phosphate derivatives), and plant hormones (such as, gibberellic or abscisic acid).

"Specifically regulatable" refers to the ability of a small molecule to preferentially affect transcription from one promoter or group of promoters (e.g., the α -amylase gene family), as opposed to non-specific effects, such as, enhancement or reduction of global transcription within a cell by a small molecule.

"Seed maturation" or "grain development" refers to the period starting with fertilization in which metabolizable reserves, e.g., sugars, oligosaccharides, starch, phenolics, amino acids, and proteins, are deposited, with and without vacuole targeting, to various tissues in the seed (grain), e.g., endosperm, testa, aleurone layer, and scutellar epithelium, leading to grain enlargement, grain filling, and ending with grain desiccation.

"Inducible during seed maturation" refers to promoters which are turned on substantially (greater than 25%) during seed maturation.

"Heterologous" is defined to mean not identical, e.g. different in nucleotide and/or amino acid sequence, phenotype or an independent isolate.

"Heterologous DNA" or "foreign DNA" refers to DNA which has been introduced into plant cells from another source, or which is from a plant source, including the same plant source, but which is under the control of a promoter or terminator that does not normally regulate expression of the heterologous DNA.

"Heterologous protein" is a protein, including a polypeptide, encoded by a heterologous DNA. A "transcription regulatory region" or "promoter" refers to nucleic acid sequences that influence and/or promote initiation of transcription. Promoters are typically considered to include regulatory regions, such as enhancer or inducer elements.

"Chimeric" is defined to mean the linkage of two or more DNA sequences which are derived from different sources, strains or species, i.e., from bacteria and plants, or that two or more DNA sequences from the same species are linked in a way that does not occur

in the native genome. Thus, the DNA sequences useful in the present invention may be naturally occurring, semi-synthetic or entirely synthetic. The DNA sequence may be linear or circular, Le, may be located on an intact or linearized plasmid, such as the binary plasmids described below.

A "chimeric gene," in the context of the present invention, typically comprises a promoter sequence operably linked to DNA sequence that encodes a heterologous gene product, e.g., a selectable marker gene or a fusion protein gene. A chimeric gene may also contain further transcription regulatory elements, such as transcription termination signals, as well as translation regulatory signals, such as, termination codons.

"Operably linked" refers to components of a chimeric gene or an expression cassette that function as a unit to express a heterologous protein. For example, a promoter operably linked to a heterologous DNA, which encodes a protein, promotes the production of functional mRNA corresponding to the heterologous DNA.

A "product" encoded by a DNA molecule includes, for example, RNA molecules and polypeptides.

"Removal" in the context of a metabolite includes both physical removal as by washing and the depletion of the metabolite through the absorption and metabolizing of the metabolite by the cells.

"Substantially isolated" is used in several contexts and typically refers to the at least partial purification of a protein or polypeptide away from unrelated or contaminating components.

Methods and procedures for the isolation or purification of proteins or polypeptides are known in the art.

"Stably transformed" as used herein refers to a cereal cell or plant that has foreign nucleic acid stably integrated into its genome which is transmitted through multiple generations.

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1. The first step is to identify the problem or question that needs to be addressed. This involves understanding the context and the specific requirements of the task.

7.4.1 GENERAL APPROACH

Generally, the nomenclature and laboratory procedures with respect to standard recombinant DNA technology can be found in Sambrook, et al., MOLECULAR - CLONING - A LABORATORY MANUAL, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1989 and in S.B. Gelvin and R.A. Schilperoort, PLANT MOLECULAR BIOLOGY, 1988. Other general references are provided throughout this document. The procedures therein are known in the art and are provided for the convenience of the reader.

Most of the recombinant DNA methods employed in practicing the present invention are standard procedures, well known to those skilled in the art, and described in detail in, or example, European Patent Application Publication Number 223,452, published November 29, 1986, which is incorporated herein by reference. Enzymes are obtained from commercial sources and are used according to the vendor's recommendations or other variations known in the art. General references containing such standard techniques include the following: R. Wu, ed. (1979) Methods Emzymology, Vol. 68; J.H. Miller (1972) Experiments in Molecular Genetics; J. Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual 2nd Ed.; D.M. Glover, ed. (1985) DNA Cloning Vol. II; H.G. Polites and K.R. Marotti (1987) "A step-wise protocol for cDNA synthesis, " Biotechniques 4; 514-520; S.B. Gelvin and R.A. Schilperoort, eds. Introduction, Expression, and Analysis of Gene Products in Plants, all of which are incorporated by reference.

7.4.2 CONTROL OF PLANT GENE EXPRESSION

7.4.2.1 Using a Transiently-active Promoter

This invention relates to a method of creating transgenic plants wherein the expression of certain plant traits is ultimately under external control. In one embodiment the control is achieved through application of an external stimulus; in another embodiment it is achieved through hybridization, in still another embodiment it is achieved by direct introduction of a recombinase or recombinase gene into a plant. The transgenic plants of the present invention are prepared by introducing into their genome a series of functionally interrelated DNA sequences, containing the following basic elements: a plant-active promoter that is active at a particular stage in plant development or under particular environmental conditions ("transiently- active promoter"), a gene whose expression results in an altered plant phenotype which is linked to the transiently-active promoter through a blocking sequence separating the transiently-active promoter and the gene, unique specific excision sequences flanking the blocking sequence, wherein the specific excision sequences are recognizable by a site- specific recombinase, a gene encoding the site-specific recombinase, an alternative repressible promoter linked to the recombinase gene, and an alternative gene that encodes the repressor specific for the repressible promoter, the action of the repressor being responsive to an applied or exogenous stimulus. While these elements may be arranged in any order that achieves the interactions described below, in one embodiment they are advantageously arranged as follows: a first DNA sequence contains the transiently-active promotor, a first specific excision sequence, the blocking sequence, a second specific excision sequence, and the gene whose expression results in an altered plant phenotype; a second DNA sequence contains the repressible promoter operably linked to the recombinase gene, and optionally an enhancer; and a third DNA sequence containing the gene encoding the repressor specific for the repressible promoter, itself linked to a promoter functional and constitutive in plants. The third DNA sequence can conveniently act as the blocking sequence located in the first DNA sequence, but can also occur separately without altering the function of the system. This embodiment can be modified such that the recombinase sequence is introduced separately via a viral vector. In an alternative embodiment, an advantageous arrangement is as follows: a first plant containing a DNA sequence comprising the transiently-active promotor, a first specific excision signal sequence, the blocking sequence, a second specific excision signal sequence, and the gene whose expression results in an altered plant phenotype; a second

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these cells are the genes for the recombinase, linked to a repressible promoter, and the gene coding for the repressor. The repressor is expressed constitutively and represses the expression of the recombinase. These plant cells can be regenerated into a whole plant and allowed to produce seed. The mature seed is exposed to a stimulus, such as a chemical agent, that inhibits the function of the repressor. Upon inhibition of the repressor, the promoter driving the recombinase gene is depressed and the recombinase gene is expressed. The resulting recombinase recognizes the specific excision sequences flanking the blocking sequence, and effects the removal of the blocking sequence. The late embryogenesis promoter and the lethal gene are then directly linked. The lethal gene is not expressed, however, because the promoter is not active at this time in the plant's life cycle. This seed can be planted, and grown to produce a desired crop of plants. As the crop matures and produces a second generation of seed, the late embryogenesis promoter becomes active, the lethal gene is expressed in the maturing second generation seed, which is rendered incapable of germination. In this way, accidental reseeding, escape of the crop plant to areas outside the area of cultivation, or germination of stored seed can be avoided.

7.4.2.3 Transgenic Plants Hybridized to Display Phenotype Not Seen in Either Parent

In an alternative preferred embodiment, the present invention involves a pair of transgenic plants that are hybridized to produce progeny that display a phenotype not seen in either parent. In this alternative embodiment a transiently-active promoter that is active only in late embryogenesis can be linked to a lethal gene, with an intervening blocking sequence bounded by the specific excision sequences. These genetic sequences can be introduced into plant cells to produce one transgenic parent plant. The recombinase gene is linked to a germination-specific promoter and introduced into separate plant cells to produce a second transgenic parent plant. Both of these plants can produce viable seed if pollinated. If the first and second transgenic parent plants are hybridized, the progeny will contain both the blocked lethal gene and the recombinase gene. The recombinase is expressed upon germination of the seed and effects the removal of the blocking sequence, as in the first embodiment, thereby directly linking the lethal gene and the transiently-active promoter. As in the first embodiment, this promoter becomes active during maturation of the second generation seed, resulting in seed that is incapable of germination. Ideally, the first parent employs a male-sterility gene as the blocking sequence, and includes an herbicide resistance gene. In this way, self-pollination of the

first transgenic parent plant is avoided, and self-pollinated second transgenic parent plants can be eliminated by application of the herbicide. In the hybrid progeny, the male-sterility gene is removed by the recombinase, resulting in hybrid progeny capable of self-pollination.

7.4.2.3.1 Linking the Recombinase Gene to an Inducible Promoter

In another embodiment, the recombinase gene is linked to an inducible promoter. Examples of such promoters include the copper, controllable gene expression system (Mett et al., 1993) and the steroid- inducible gene system (Schena et al., 1991). Exposure of the transgenic plant to the inducer specific for the inducible promoter leads to expression of the recombinase gene and the excision of the blocking sequence. The gene that results in an altered plant phenotype is then expressed when the transiently active promoter becomes active.

7.4.2.3.2 Selection of an Appropriate Promoter

Any appropriate transiently-active promoter can be used, and selection of an appropriate promoter will be governed by such considerations as plant type and the phenotypic trait over which control is sought. The transiently-active promoter is preferably not a "leaky" promoter, meaning that it is active substantially only during a well- defined phase of plant growth or under particular environmental conditions, and substantially inactive at all other times. This property prevents the premature "triggering" of the system. There are numerous published examples of transiently-active promoters, which can be applied in the present system. The principle consideration for selecting an appropriate promoter is the stage in the plant's life at which it is desired to have the altered phenotype expressed. If it is desired to have the phenotype expressed after the first generation, a promoter that is active during seed production is preferred, as it will not be active during the vegetative phase of first generation plant growth. If it is desired to have the altered phenotype expressed at some time during the first generation itself, a promoter that is active at an earlier stage would be appropriate. It will be readily apparent to workers conversant in the art that the timing of the application of the external stimulus to the plant to trigger the system, in those embodiments employing the repressible promoter system, should occur prior to the stage at which the selected transiently-active promoter is active for the generation of plant which is desired to display the altered phenotype. A promoter

active in late embryogenesis, such as the LEA promoter, Hughes and Galau, 1989 and 1991, Galau, et al., 1991, 1992 and 1993, is ideal when it is desired to have the altered phenotype appear after the first generation, because it is active only during the formation of the embryo within the seed, after the first generation plant has completed a season of vegetative growth (embryogenesis is virtually the last stage in seed formation, after most other fruit and seed structures are formed).

7.4.2.3.3 Gene(s) Linked to the Plant Development Promoter

The gene or genes linked to the plant development promoter can be any gene or genes whose expression results in a desired detectable phenotype. This phenotype could be any trait that would be desired in a plant in one situation, but not desired in another, such as male sterility, drought resistance, insect resistance, early or late seed germination, or early or late flowering, to give a few examples. Often a plant can possess traits that are advantageous in some ways or under some conditions, but at a certain cost to the plant. For instance, a trait for insect resistance might involve the production of secondary plant metabolites or structures, at a certain metabolic expense to the plant. This is advantageous in an environment where pests are present, but essentially an unnecessary-burden where they are not. Another example is the production of seeds in an annual fruit crop, such as watermelon. Obviously, it is necessary for at least one generation of plants to produce seeds, so that a seed company can produce seed for sale to growers, but a seedless fruit crop grown from that seed is commercially desirable. Still another example is a trait that allows ready and rapid seed germination in a cereal crop. This is advantageous for getting a crop established as rapidly as possible and with a minimum of effort, but very undesirable if it leads to germination of the harvested grain in the grain bin. Still another example would be where the plant is desirable in one location or season (as a winter forage crop, for instance), but considered a weed in another. If the second generation seed were incapable of germination, it would prevent post-harvest germination, the "tescape" of a plant through natural seed dispersal into a location where it is not desired, or accidental reseeding. These last two examples could advantageously employ a lethal gene (meaning a gene whose expression somehow interferes in plant growth or development), so that the second generation seed simply will not germinate, or the last example could alternatively employ any gene that introduces a trait that decreases the plant's vigor, such as disease susceptibility, early flowering, low seed production, or seedless. A ribosomal

inhibitor protein ("RIP") gene is a preferred lethal gene, the saponin 6 RIP, (GenBank ID SOSAPG, Accession No. X15655), being particularly preferred. RIP directly interferes in the expression of all protein in a plant cell, without being toxic to other organisms.

Expression of RIP in the cells of the embryo would entirely prevent germination of the seed.

7.4.2.3.4 Blocking Sequence

The blocking sequence can be any sequence that prevents expression of the gene linked to the transiently-active promotor, such as a termination signal, but in those embodiments employing a repressible promoter is advantageously the sequence that codes for the repressor. In this way, when the blocking sequence is excised, the repressor gene is eliminated, thus further minimizing the chance of later inhibition of the system. In the hybrid embodiment, the blocking sequence is advantageously a gene that produces male sterility (such as a lethal gene linked to an anther-specific promotor). In this way, hybridization is facilitated, but hybrid progeny will be capable of self-pollination when the blocking sequence is removed by the recombinase.

7.4.2.3.5 Repressible Promoter System

In those embodiments employing a repressible promoter system, the gene encoding the repressor is responsive to an outside stimulus, or encodes a repressor element that is itself responsive to an outside stimulus, so that repressor function can be controlled by the outside stimulus. The stimulus is preferably one to which the plant is not normally exposed, such as a particular chemical, temperature shock, or osmotic shock. In this way, the simple application of the stimulus will block the repression of the recombinase, yet there will be a low probability of the repressor being accidentally or incidentally blocked. If the repressor is sensitive to a chemical stimulus, the chemical is preferably non-toxic to the crop and to non-pest animals. A preferred system is the Tn10 tet repressor system, which is responsive to tetracycline. Gatz and Quail (1988); Gatz, et al. (1992). In this system, a modified Cauliflower Mosaic Virus (CaMV) 35S promoter containing one or more, preferably three, tet operons is used; the Tn10 tet repressor gene produces a repressor protein that binds to the tet operon(s) and prevents the expression of the gene to which the promoter is linked. The presence of tetracycline inhibits binding of the Tn10 tet repressor to the tet operon(s), allowing free expression of the linked gene. This system is

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7.4.3.1 Isolation of a Viral Gene

Several different courses exist to isolate a viral gene. To do so, one having ordinary skill in the art can use information about the genomic organization of potyviruses, cucumoviruses or comoviruses to locate and isolate the coat protein gene or the nuclear inclusion body genes. The coat protein gene in potyviruses, is located at the 3' end of the RNA, just prior to a stretch of about 200-300 adenine nucleotide residues. The nuclear inclusion body B (NIb) gene is located just 5' to the coat protein gene, and the nuclear inclusion body A (NIa) gene is 5' to the NIb gene. Additionally, the information related to proteolytic cleavage sites is used to determine the N- terminus of the potyvirus coat protein gene and the N- and C-terminus of non-coat protein genes. The protease recognition sites are conserved in the potyviruses and have been determined to be either the dipeptide Gln-Ser, Gln-Gly or Gln-Ala. The nucleotide sequences which encode these dipeptides can be determined.

Using methods well known in the art, a quantity of virus is grown and harvested. The viral RNA is then separated and the viral gene isolated using a number of known procedures. A cDNA library is created using the viral RNA, by methods known to the art. The viral RNA is incubated with primers that hybridize to the viral RNA and reverse transcriptase, and a complementary DNA molecule is produced. A DNA complement of the complementary DNA molecule is produced and that sequence represents a DNA copy (cDNA) of the original viral RNA molecule. The DNA complement can be produced in a manner that results in a single double stranded cDNA or polymerase chain reactions can

be used to amplify the DNA encoding the cDNA with the use of oligomer primers specific for the viral gene. These primers can include in addition to viral specific sequences, novel restriction sites used in subsequent cloning steps.

Thus, a double stranded DNA molecule is generated which contains the sequence information of the viral RNA. These DNA molecules can be cloned in *E. coli* plasmid vectors after the additions of restriction enzyme linker molecules by DNA ligase. The various fragments are inserted into cloning vectors, such as well-characterized plasmids, which are then used to transform *E. coli* to create a cDNA library.

Since potyvirus genes are generally conserved, oligonucleotides based on an analogous gene from a previous isolate or an analogous gene fragment from a previous isolate can be used as a hybridization probe to screen the cDNA library to determine if any of the transformed bacteria contain DNA fragments with the appropriate viral sequences. The cDNA inserts in any bacterial colonies which hybridize to these probes can be sequenced. The viral gene is present in its entirety in colonies which have sequences that extend 5' to sequences which encode a N-terminal proteolytic cleavage site and 3' to sequences which encode a C-terminal proteolytic cleavage site for the gene of interest.

Alternatively, cDNA fragments may be inserted in the sense orientation into expression vectors. Antibodies against a viral protein may be used to screen the cDNA expression library and the gene can be isolated from colonies which express the protein.

7.4.3.3 Table of Selected Literature References to Methods of Isolating, cloning and Expressing Viral Genes

The nucleotide sequences encoding the coat protein genes and nuclear inclusion genes of a number of viruses have been determined and the genes have been inserted into expression vectors. The expression vectors contain the necessary genetic regulatory sequences for expression of an inserted gene. The coat protein gene is inserted such that those regulatory sequences are functional and the genes can be expressed when incorporated into a plant genome. Selected literature references to methods of isolating, cloning and expressing viral genes are listed on Table 3, below.

TABLE 3.
Cloned Genes From RNA Viruses

Viral Gene	Reference
Papaya ringspot cp	M.M. Fitch et al., Bio/Technology, 10, 1466(1992)
Potato virus X cp	K. Ling et al., Bio/Technology, 2, 752 (1991); A. Hoekema et al., Bio/Technology, 7, 273 (1989)
Watermelon Mosaic Virus II cp	H. Quemada et al., J. Gen. Virol., 71, 1451 (1990); S. Namba et al., Phytopathology, 82, 940 (1992)
Zucchini yellow MosaicVirus cp	S. Namba et al., Phytopathology, 82, 940 (1992)
Tobacco Mosaic Virus cp	R.S. Nelson et al., Bio/Technology, 6, 403 (1988); P. Powell Abel et al., Science, 232, 738 (1986)
Alfalfa Mosaic Virus cp	Loesch-Fries et al., EMBO J., 6, 1845 (1987); N.E. Turner et al., EMBO J., 6, 1181 (1987)
Soybean Mosaic Virus cp	D.M. Stark et al., Biotechnology, 7, 1257 (1989)
Cucumber Mosaic Virus strain C cp	H.Q. Quemada et al., Molec. Plant Pathol., 81, 794 (1991)
Cucumber Mosaic Virus strain WL cp	UpJohn Co. (PCT W090/02185)
Tobacco etch virus cp	Allison et al., Virology, 147, 309 (1985)
Tobacco etch virus nuclear inclusion protein	J.C. Carrington et al., J. Virol., 61, 2540 (1987)
Pepper Mottle Virus cp	W.G. Dougherty et al., Virology, 146, 282 (1985)
Potato virus Y cp	D.D. Shukla et al., Virology, 152, 118 (1986)

germinating seeds. In the present invention, particle bombardment is the preferred transformation procedure.

The construct also includes a gene encoding a mature heterologous protein in a form suitable for secretion from plant cells. The gene encoding the recombinant heterologous protein is placed under the control of a metabolically regulated promoter. Metabolically regulated promoters are those in which mRNA synthesis or transcription, is repressed or upregulated by a small metabolite or hormone molecule, such as the rice RAmy3D and RAmy3E promoters, which are upregulated by sugar-depletion in cell culture. For protein production in germinating seeds from regenerated transgenic plants, a preferred promoter is the-Ramy 1A promoter, which is up-regulated by gibberellic acid during seed germination. The expression construct also utilizes additional regulatory DNA sequences e.g., preferred codons, termination sequences, to promote efficient translation of AAT, as will be described.

7.4.5 DISEASE RESISTANCE

One aspect of the present invention relates to the use of trait DNA molecules which are heterologous to the plant -- e.g., DNA molecules that confer disease resistance to plants transformed with the DNA construct. The present invention is useful in plants for imparting resistance to a wide variety of pathogens including viruses, bacteria, fungi, viroids, phytoplasmas, nematodes, and insects. The present invention may also be used in mammals to impart genetic traits. Resistance, inter alia, to the following viruses can be achieved by the method of the present invention: tomato spotted wilt virus, impatiens necrotic spot virus, groundnut ringspot virus, potato virus Y, potato virus X, tobacco mosaic virus, turnip mosaic virus, tobacco etch virus, papaya ringspot virus, tomato mottle virus, tomato yellow leaf curl virus, or-combinations thereof. Resistance, inter alia, to the following bacteria can also be imparted to plants in accordance with present invention: *Pseudomonas solanacearum*, *Pseudomonas syringae* pv. *tabaci*, *Xanthomonas campestris* pv. *pelargonii*, and *Agrobacterium* *tumefaciens*. Plants can be made resistant, inter alia, to the following fungi by use of the method of the present invention: *Fusarium oxysporum* and *Phytophthora infestans*. Suitable DNA molecules include a DNA molecule encoding a coat protein, a replicase, a DNA molecule not encoding protein, a DNA molecule encoding a viral gene product, or combinations thereof.

7.4.5.1 Sense/Antisense orientation

The DNA molecule conferring disease resistance can be positioned within the DNA construct in sense orientation. Alternatively, it can have an antisense orientation. Antisense RNA technology involves the production of an RNA molecule that is complementary to the messenger RNA molecule of a target gene; the antisense RNA can potentially block all expression of the targeted gene. In the anti-virus context, plants are made to express an antisense RNA molecule corresponding to a viral RNA (that is, the antisense RNA is an RNA molecule which is complementary to a plus sense RNA species encoded by an infecting virus). Such plants may show a slightly decreased susceptibility to infection by that virus. Such a complementary RNA molecule is termed antisense RNA.

7.4.6 OTHER TRAITS

The present invention is also used to confer traits other than disease resistance on plants. For example, DNA molecules which impart a plant genetic trait can be used as the DNA trait molecule of the present invention. In this aspect of the present invention, suitable trait DNA molecules encode for desired color, enzyme production, or combinations thereof.

7.4.6.1 GENE SILENCING

The silencer DNA molecule of the present invention can be selected from virtually any nucleic acid which effects gene silencing. This involves the cellular mechanism to degrade mRNA homologous to the transgene mRNA. The silencer DNA molecule can be heterologous to the plant, need not interact with the trait DNA molecule in the plant, and can be positioned 3' to the trait DNA molecule. For example, the silencer DNA molecule can be a viral cDNA molecule, a jellyfish green fluorescence protein encoding DNA molecule, a plant DNA molecule, or combinations thereof.

While not wishing to be bound by theory, by use of the construct of the present invention, it is believed that post-transcriptional gene silencing is achieved. More particularly, the silencer DNA molecule is believed to boost the level of heterologous RNA within the cell above a threshold level. This activates the degradation mechanism by which viral resistance is achieved.

7.4.6.1.1 TRAIT & SILENCER DNA MOLECULES ENCODING RNA MOLECULES - TRANSLATABLE

It is possible for the DNA construct of the present invention to be configured so that the trait and silencer DNA molecules encode RNA molecules which are translatable. As a result, that RNA molecule will be translated at the ribosomes to produce the protein encoded by the DNA construct. Production of proteins in this manner can be increased by joining the cloned gene encoding the DNA construct of interest with synthetic double-stranded oligonucleotides which represent a viral regulatory sequence (i.e., a 5' untranslated sequence). See U.S. Patent No. 4,820,639 to Gehrke and U.S. Patent No. 5,849,527 to Wilson which are hereby incorporated by reference.

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7.4.7 RECOMBINANT DNA TECHNOLOGY

The subject DNA construct can be incorporated in cells using conventional recombinant DNA technology. Generally, this involves inserting the DNA construct into an expression system to which the DNA construct is heterologous (i.e. not normally present). The heterologous DNA construct may be inserted into the expression system or vector in proper sense orientation and correct reading frame. The vector contains the necessary elements for the transcription of the inserted sequences.

7.4.7.1 Restriction enzyme cleavage and ligation

U.S. Patent No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture.

7.4.7.2 Introduction to viruses

Recombinant genes may also be introduced into viruses, such as vaccinia virus. Recombinant viruses can be generated by transfection of plasmids into cells infected with virus.

7.4.9 PLANT EXPRESSION VECTOR PRODUCTION OF MATURE PROTEINS IN PLANTS

7.4.9.1 SUITABLE VECTORS

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pER322, pBR325, pACYC177, pACYC1084, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see F.W. Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," Gene Expression Technology vol. 185 (1990), which is hereby incorporated by reference), and any derivatives thereof. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1989), which is hereby incorporated by reference.

7.4.9.2 HOST-VECTOR SYSTEMS

A variety of host-vector systems may be utilized to carry out the present invention. Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression elements of these vectors vary in their strength and specificities. Depending upon the host- vector system utilized, any one of a number of suitable transcription and, perhaps, translation elements can be used.

7.4.10 GENETIC SIGNALS AND PROCESSING EVENTS

In order to express the viral gene, the necessary genetic regulatory sequences must be provided. Since the proteins encoded in a potyvirus, genome are produced by the post translational processing of a polyprotein, a viral gene isolated from viral RNA does not contain transcription and translation signals necessary for its expression once transferred and integrated into a plant genome. It must, therefore, be engineered to contain a plant expressible promoter, a translation initiation codon (ATG) and a plant functional poly(A) addition signal (AATAAA) 3' of its translation termination codon. In the present invention, a viral gene is inserted into a vector which contains cloning sites for insertion 3' of the initiation codon and 5' of the poly(A) signal. The promoter is 5' of the initiation codon such that when structural genes are inserted at the cloning site, a functional unit is formed in which the inserted genes are expressed under the control of the various genetic regulatory sequences.

Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

7.4.10.1 Signal Sequences for production of mature proteins

In addition to encoding the protein of interest, the chimeric gene encodes a signal sequence (or signal peptide) that allows processing and translocation of the protein, as appropriate. Suitable signal sequences are described in above-referenced PCT application WO 95/14099. The plant signal sequence is placed in frame with a heterologous nucleic acid encoding a mature protein, forming a construct which encodes a fusion protein having an N- terminal region corresponding to the signal peptide and, immediately adjacent to the C- terminal amino acid of the signal peptide, the N-terminal amino acid of the mature heterologous protein. The expressed fusion protein is subsequently secreted and processed by signal peptidase cleavage precisely at the junction of the signal peptide and the mature protein, to yield the mature heterologous protein.

In another embodiment of the invention, the coding sequence in the fusion protein gene, in at least the coding region for the signal sequence, may be codon-optimized for optimal expression in plant cells, e.g., rice cells, as described below.

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Transcription of DNA is dependent upon the presence of a promoter which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eucaryotic promoters differ from those of procaryotic promoters. Furthermore, eucaryotic promoters and accompanying genetic signals may not be recognized in or may not function in a procaryotic system, and, further, procaryotic promoters are not recognized and do not function in eucaryotic cells.

The segment of DNA referred to as the promoter is responsible for the regulation of the transcription of DNA into mRNA. A number of promoters which function in plant cells are known in the art and may be employed in the practice of the present invention. These promoters may be obtained from a variety of sources such as plants or plant viruses, and may include but are not limited to promoters isolated from the caulimovirus group such as the cauliflower mosaic virus 35S promoter (CaMV35S), the enhanced cauliflower mosaic virus 35S promoter (enh CaMV35S), the figwort mosaic virus full-length transcript promoter (FMV35S), and the promoter isolated from the chlorophyll alb binding protein. Other useful promoters include promoters which are capable of expressing the potyvirus proteins in an inducible manner or in a tissue-specific manner in certain cell types in which the infection is known to occur. For example, the inducible promoters from phenylalanine ammonia lyase, chalcone synthase, hydroxyproline rich glycoprotein, extensin, pathogenesis-related proteins (e.g. PR-1a), and wound-inducible protease inhibitor from potato may be useful.

Preferred promoters for use in the present viral gene expression cassettes include the constitutive promoters from CaMV, the Ti genes nopaline synthase (Bevan et al., Nucleic Acids Res. II, 369-385 (1983)) and octopine synthase (Depicker et al., J. Mol. Appl. Genet., 1, 561- 564 (1982)), and the bean storage protein gene phaseolin. The poly(A) addition signals from these genes are also suitable for use in the present cassettes. The particular promoter selected is preferably capable of causing sufficient expression of the DNA coding sequences to which it is operably linked, to result in the production of amounts of the proteins or the RNAs effective to provide viral resistance, but not so much as to be detrimental to the cell in which they are expressed. The promoters selected should

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be capable of functioning in tissues including but not limited to epidermal, vascular, and mesophyll tissues. The actual choice of the promoter is not critical, as long as it has sufficient transcriptional activity to accomplish the expression of the preselected proteins or antisense RNA, and subsequent conferral of viral resistance to the plants.

Promoters vary in their "strength" (i.e. their ability to promote transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promoters in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promoters may be used. For instance, when cloning in *E. coli*, its bacteriophages, or plasmids, promoters such as the T7 phage promoter, lac promoter, trp promoter, recA promoter, ribosomal RNA promoter, the P_R and P_L promoters of coliphage lambda and others, including but not limited, to lacUV5, ompF, bla, lpp, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid trp-lacUV5 (tac) promoter or other *E. coli* promoters produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene.

7.4.10.2.1 Promoters that transcribe the cereal α -amylase genes and sucrose synthase genes

The transcription regulatory or promoter region is chosen to be regulated in a manner allowing for induction under selected cultivation conditions, e.g., sugar depletion in culture or water uptake followed by gibberellic acid production in germinating seeds. Suitable promoters, and their method of selection are detailed in above-cited PCT application WO 95/14099. Examples of such promoters include those that transcribe the cereal α -amylase genes and sucrose synthase genes, and are repressed or induced by small molecules, like sugars, sugar depletion or phytohormones such as gibberellic acid or abscisic acid. Representative promoters include the promoters from the rice α -amylase RAm1A, RAm1B, RAm2A, RAm3A, RAm3B, RAm3C, RAm3D, and RAm3E genes, and from the pM/C, gKAm141, gKAm155, Amy32b, and HV18 barley (α -amylase genes. These promoters are described, for example, in ADVANCES IN PLANT BIOTECHNOLOGY Ryu, D.D.Y., et al, Eds., Elsevier, Amsterdam, 1994, p.37, and references cited therein. Other suitable promoters include the sucrose synthase and sucrose-6-phosphate-synthetase (SPS) promoters from rice and barley.

Other suitable promoters include promoters which are regulated in a manner allowing for induction under seed-maturation conditions. Examples of such promoters include those associated with the following monocot storage proteins: rice glutelins, oryzins, and prolamines, barley hordeins, wheat gliadins and glutelins, maize zeins and glutelins, oat glutelins, and sorghum kafirins, millet pennisetins, and rye secalins.

A preferred promoter for expression in germinating seeds is the rice α -amylase RAmy1A promoter, which is upregulated by gibberellic acid. Preferred promoters for expression in cell culture are the rice α -amylase RAmy3D and RAmy3E promoters which are strongly upregulated by sugar depletion in the culture. These promoters are also active during seed germination. A preferred promoter for expression in maturing seeds is the barley endosperm-specific BI-hordein promoter (Brandt, A., et al., (1985) Carlsberg Res. Commun. 50:333-345).

The chimeric gene may further include, between the promoter and coding sequences, the 5' untranslated region (5' UTR) of an inducible monocot gene, such as the 5' UTR derived from one of the rice or barley α -amylase genes mentioned above. One preferred 5' UTR is that derived from the RAmy1A gene, which is effective to enhance the stability of the gene transcript.

7.4.10.2.2 Use of inducers

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promoter unless specifically induced. In certain operations, the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the lac operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as trp, pro, etc., are under different controls.

7.4.10.3 TRANSCRIPTION INITIATION SIGNALS

Specific initiation signals are also required for efficient gene transcription in procaryotic cells.

These transcription initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promoter, may also contain any combination of various "strong" transcription initiation signals.

7.4.10.4 Translation dependent on Shine-Dalgarno sequence (in procaryotes)

7.4.10.5 Naturally-Occurring, Heterologous Protein Coding Sequences

7.4.10.5.3 (iii) Human serum albumin: Mature HSA as found in human serum is composed of 585 amino acids. The protein has no N-linked glycosylation sites.

7.4.10.5.4 (iv) Subtilisin BPN': Native proBPN' as produced in *B. ainyloliquefaciens* is composed of 352 amino acids. The proBPN' polypeptide contains a 77 amino acid "pro" moiety. The remainder of the polypeptide, which forms the mature active BPN', is a 275 amino acid sequence. Native l3PN' as produced in *Bacillus* is not glycosylated.

7.4.10.6 A4. Codon-Optimized Coding Sequences

In accordance with one aspect of the invention, it has been discovered that a severalfold enhancement of expression level can be achieved in plant cell culture by modifying the native coding sequence of a heterologous gene by contain predominantly or exclusively, highest-frequency codons found in the plant cell host.

The method will be illustrated for expression of a heterologous gene in rice plant cells, it being recognized that the method is generally applicable to any monocot. As a first step, a representative set of known coding gene sequence from rice is assembled. The sequences are then analyzed for codon frequency for each amino acid, and the most frequent codon is selected for each amino acid. This approach differs from earlier reported codon matching methods, in which more than one frequent codon is selected for at least some of the amino acids. The optimal codons selected in this manner for rice and barley are shown in Table 4.

TABLE 4

Amino Acid	Rice Preferred Codon	Barley Preferred Codon
Ala A	GCC	
Arg R	CGC	

Asn N	AAC	
Asp D	GAC	
Cys C	UGC	
Gln Q	CAG	
Glu E	GAG	
Gly G	GGC	
His H	CAC	
Ile I	AUC	
Leu L	CUC	
Lys K	AAG	
Phe F	UUC	
Pro P	CCG	CCC
Ser S	AGC	UCC
Thr T	ACC	
Tyr Y	UAC	
Val V	GUC	GUG
stop	UAA	UGA

In a preferred embodiment, the BPN' coding sequence is further modified to eliminate potential N-glycosylation sites, as native BPN' is not glycosylated. Table 5 illustrates preferred codon substitutions, which eliminate all potential N-glycosylation sites in subtilisin BPN'.

N-Glycosylation Sites	Location (Asn) (in mature protein)	Amino Acid Substitution
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Asn Asn Ser	61	Thr Asn Ser
Asn Asn Ser	76	Thr Asn Ser
Asn Met Ser	123	Thr Met Ser
Asn Gly Thr	218	Ser Gly Thr'
Asn Trp Thr	240	Thr Trp Thr

'improved thermostability; Bryan, et al., Proteins: Structure, Function, and Genetics 1:326 (1986).

7.4.10.7 TERMINATION SEQUENCE COUPLED TO THE FUSION END

The present invention can also utilize a termination sequence operatively coupled to the fusion gene to end transcription. Suitable transcription termination sequences include the termination region of a 3' non-translated region. This will cause the termination of transcription and the addition of polyadenylated ribonucleotides to the 3' end of the transcribed mRNA sequence. The termination region or 31 non-translated region will be additionally one of convenience. The termination region may be native with the promoter region or may be derived from another source, and preferably includes a terminator and a sequence coding for polyadenylation. Suitable 3' non-translated regions include but are not limited to: (1) the 3' transcribed, non-translated regions containing the polyadenylated signal of Agrobacterium tumor-inducing (Ti) plasmid genes, such as the nopaline synthase (NOS) gene or the 35S promoter terminator gene, and (2) plant genes like the soybean 7S storage protein genes and the pea small subunit of the ribulose 1,5-bisphosphate carboxylase-oxygenase (ssRUBISCO) E9 gene.

The termination region or 3' non-translated region which is employed is one which will cause the termination of transcription and the addition of polyadenylated ribonucleotides to the 3' end of the transcribed mRNA sequence. The termination region may be native with the promoter region, native with the structural gene, or may be derived from another source, and preferably include a terminator and a sequence coding for polyadenylation. Suitable 3' non-translated regions of the chimeric plant gene include but are not limited to: (1) the 3' transcribed, non-translated regions containing the polyadenylation signal of Agrobacterium tumor-inducing (Ti) plasmid genes, such as the nopaline synthase (NOS) gene, and (2) plant genes like the soybean 7S storage protein genes.

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7.4.10.8 Transcription and Translation Terminators for production of mature proteins

The chimeric gene may also include, downstream of the coding sequence, the 3' untranslated region (Y UTR) from an inducible monocot gene, such as one of the rice or barley α -amylase genes mentioned above. One preferred 3' UTR is that derived from the RAmYL A gene. This sequence includes non-coding sequence 5' to the polyadenylation site, the polyadenylation site, and the transcription termination sequence. The transcriptional termination region may be selected, particularly for stability of the mRNA to enhance expression. Polyadenylation tails (Alber and Kawasaki, 1982, Mol. and Appl. Genet. 1:419-434) are also commonly added to the expression cassette to optimize high levels of transcription and proper transcription termination, respectively. Polyadenylation sequences include but are not limited to the Agrobacterium octopine synthetase signal (Gielen, et al., EMBO J. 3:835- 846 (1984) or the nopaline synthase of the same species (Depicker, et al. , Mol. Appl. Genet. 1:561- 573 (1982).

7.4.11 SELECTABLE MARKER GENE

Selectable marker genes may be incorporated into the present expression cassettes and used to select for those cells or plants which have become transformed. The marker gene employed may express resistance to an antibiotic, such as kanamycin, gentamycin, G418, hygromycin, streptomycin, spectinomycin, tetracycline, chloramphenicol, and the like.

Other markers could be employed in addition to or in the alternative, such as, for example, a gene coding for herbicide tolerance such as tolerance to glyphosate, sulfonylurea, phosphinothricin, or bromoxynil. Additional means of selection could include resistance to methotrexate, heavy metals, complementation providing prototrophy to an auxotrophic host, and the like.

For example, see Table 1 of PCT WO/91/10725, cited above. The present invention also envisions replacing all of the virus-associated genes with an array of selectable marker genes.

The particular marker employed will be one which will allow for the selection of transformed cells as opposed to those cells which were not transformed. Depending on the number of different host species one or more markers may be employed, where different conditions of selection would be useful to select the different host, and would be known to those of skill in the art. A screenable marker or "reporter gene" such as the 0-glucuronidase gene or luciferase gene may be used in place of, or with, a selectable marker. Cells transformed with this gene may be identified by the production of a blue product on treatment with 5-bromo-4-chloro-3-indoyl- β -D-glucuronide (X-Gluc).

In developing the present expression construct, the various components of the expression construct such as the DNA sequences, linkers, or fragments thereof will normally be insetted into a convenient cloning vector, such as a plasmid or phage, which is capable of replication in a bacterial host, such as E. coli. Numerous cloning vectors exist that have been described in the literature. After each cloning, the cloning vector may be isolated and subjected to further manipulation, such as restriction, insertion of new fragments, ligation, deletion, resection, insertion, in vitro mutagenesis, addition of

7.4.12 TRANSFERRING RECOMBINANT DNA INTO PLANT CELL

In producing transgenic plants, the DNA construct in a vector described above can be microinjected directly into plant cells by use of micropipettes to transfer mechanically the recombinant DNA. Crossway, *Mol. Gen. Genetics*, 202:179-85 (1985), which is hereby incorporated by reference. The genetic material may also be transferred into the plant cell using polyethylene glycol. Krens, et al., *Nature*, 296:72-74 (1982), which is hereby incorporated by reference.

7.4.12.2 Particle Bombardment (Biolistic Transformation)

Another approach to transforming plant cells with the DNA construct is particle bombardment (also known as biolistic transformation) of the host cell. This can be accomplished in one of several ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford et al., which are hereby incorporated by reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, a vector containing the DNA construct can be introduced into the cell by coating the particles with the vector containing that heterologous DNA construct. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried bacterial cells containing the vector and heterologous DNA construct) can also be propelled into plant cells.

7.4.12.2 Fusion of protoplasts with other entities

Yet another method of introduction is fusion of protoplasts with other entities, - either minicells, cells, lysosomes or other fusible lipid-surfaced bodies. Fraley, et al., Proc. Natl. Acad. Sci. USA, 79:1859-63 (1982), which is hereby incorporated by reference.

7.4.12.4 Electroporation

The DNA molecule may also be introduced into the plant cells by electroporation. Fromm et al., Proc. Natl. Acad. Sci. USA, 82:5824 (1985), which is hereby incorporated by reference. In this technique, plant protoplasts are electroporated in the presence of

plasmids containing the expression cassette. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and regenerate.

7.4.12.5 Infection with Agrobacterium tumefaciens or *A. rhizogenes*

Another method of introducing the DNA molecule into plant cells is to infect a plant cell with Agrobacterium tumefaciens or *A. rhizogenes* previously transformed with the gene. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots or roots, and develop further into plants. Generally, this procedure involves inoculating the plant tissue with a suspension of bacteria and incubating the tissue for 48 to 72 hours on regeneration medium without antibiotics at 25-28°C.

Agrobacterium is a representative genus of the gram-negative family Rhizobiaceae. Its species are responsible for crown gall (*A. tumefaciens*) and hairy root disease (*A. rhizogenes*). The plant cells in crown gall tumors and hairy roots are induced to produce amino acid derivatives known as opines, which are catabolized only by the bacteria. The bacterial genes responsible for expression of opines are a convenient source of control elements for chimeric expression cassettes. In addition, assaying for the presence of opines can be used to identify transformed tissue.

Heterologous genetic sequences can be introduced into appropriate plant cells, by means of the Ti plasmid of *A. tumefaciens* or the Ri plasmid of *A. rhizogenes*. The Ti or Ri plasmid is transmitted to plant cells on infection by Agrobacterium and is stably integrated into the plant genome. J. Schell, Science, 237:1176-83 (1987), which is hereby incorporated by reference.

For Agrobacterium-mediated transformation, the expression cassette will be included in a vector, and flanked by fragments of the Agrobacterium Ti or Ri plasmid, representing the right and, optionally the left, borders of the Ti or Ri plasmid transferred DNA (T-DNA). This facilitates integration of the present chimeric DNA sequences into the genome of the host plant cell. This vector will also contain sequences that facilitate replication of the plasmid in Agrobacterium cells, as well as in *E. coli* cells.

7.4.13 METHOD FOR MAKING GENETICALLY RECOMBINANT PLANTS IN COMMERCIALY FEASIBLE NUMBERS

In one preferred embodiment, the invention provides for a process for propagating plants by tissue culture in such a way as both to conserve desired plant morphology and to transform the plant with respect to one or more desired genes. The method includes the steps of (a) creating an Agrobacterium vector containing the gene sequence desired to be transferred to the propagated plant, preferably together with a marker gene; (b) taking one or more petiole explants from a mother plant and inoculating them with the Agrobacterium vector; (c) conducting callus formation in the petiole sections in culture, in the dark; and (d) culturing the resulting callus in growth medium having a benzylamino growth regulator such as benzylaminopurine or, most preferably, benzylaminopurineriboside. Additional optional growth regulators including auxins and cytokinins (indole butyric acid, benzylamine, benzyladenine, benzylaminopurine, alpha naphthylacetic acid and others known in the art) may also be present. Preferably, the petiole tissue is taken from *Pelargonium x domesticum* and the *Agrobacterium* vector contains an antisense gene for ACC synthase or ACC oxidase to prevent ACC synthase or ACC oxidase expression and, in turn, preventing ethylene formation. *Pelargoniums* propagated in culture using the present technique are resistant to wilting and petal shatter, and are morphologically conserved due to the use of petiole explants specifically and the particular culture media disclosed.

7.4.13.1 Using Petioles as the Explant Tissue

Although in theory any anatomic explants can be mixed with Agrobacterium containing the desired gene sequences to be transferred, followed by tissue culture propagation of transgenic transformed plants, in practice we have encountered unexpectedly good results using petioles as the explant tissue. We have found that morphologic conservation is virtually assured with the use of leaf petiole tissue, whereas morphologic variation-even is between two generations---can result when explants of other tissue, i.e. leaf tissue, are used. Moreover, the petiole explants should be taken from stock plants (mother plants) of which commercial propagation is desired. Commercial viability is attributable to the large number of transgenically transformed plants which can be produced from a relatively few petioles taken from the mother plant-particularly

The test tubes or vials are maintained for five days to two weeks in complete darkness, at a temperature of about 25° C. Over the five day to two week period, the section enlarges slightly and the ends form callus.

Miniature shoots start forming intermittently on the callused ends of the petiole section.

After five days to two weeks, the enlarged petiole section bearing the miniature shoots is transferred from the test tube or vial to a Magenta vial or box known in the art. The enlarged petiole sections are housed five- to-a-Magenta vial. The same growing medium as was originally charged to the test tube or vial is likewise charged to the Magenta vial, and in any event coconut milk should be present in the culture medium at this stage of the process. Also added to the medium is kanamycin (assuming the Agrobacterium contained the kanamycin resistance gene) and carbenicillin to kill any excess Agrobacterium. The Magenta vials are then maintained, under the same conditions as were the test tubes or vials, for an additional five to eight weeks in the dark and at about 25° C. The Magenta vials are then exposed to 5-10 weeks of 16 hours of light daily, in which the temperature is maintained at 72° F with 690 foot candles (6900 lux) of cool fluorescent light. During this time the petiole sections grow into enlarged clumps; the shoots elongate and turn into plantlets and many more shoots form. Once plantlets appear, they are transferred to fresh media containing kanamycin, carbenicillin and no growth hormones.

After the total growth period has elapsed, the clumps are removed and placed in sterile water. The individual plants are dissected out of the clump with a sterile scalpel. Each individual plant essentially has a series of leaves and nodes and is at least ½” high, but usually no roots are present. The individual plants are placed in RUBBER DIRT™ or other soil or soil-like growth media or growth media plugs, where rooting then takes place. Many varieties of *Pelargonium x domesticum* have been successfully tissue cultured through leaf petioles and multiplied, both with and without transgenic transformation via Agrobacterium. Morphologic variation has been minimal and within commercially

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acceptable limits for finished plant material. Other plants may be propagated by this tissue culture technique/transgenic technique also.

7.4.13.2 Creating an Agrobacterium Cell Containing the Desired Vector

The creation of the Agrobacterium cell containing the desired vector can be accomplished by means known in the art. Structural and regulatory genes to be inserted may be obtained from depositories, such as the American Type Culture Collection, Rockville, MD, 20852, as well as by isolation from other organisms, typically by the screening of genomic or cDNA libraries using conventional hybridization techniques. Typical hybridization techniques are disclosed in Sambrook, et al., Molecular Cloning--Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989). Screening may be performed by (1) nucleic acid hybridization using homologous genes or heterologous genes from other organisms, (2) probes synthetically produced to hybridize to particular sequences coding for desired protein sequences, or (3) DNA sequencing and comparison to known sequences. Sequences for specific genes may be found in various computer databases, including GenBank, National Institutes of Health, or the database maintained by the United States Patent and Trademark Office.

The genes of interest may also be identified by antibody screening of expression libraries with antibodies made against homologous proteins to identify genes encoding for homologous functions. Transposon tagging can also be used to aid the isolation of a desired gene. Transposon tagging typically involves mutation of the target gene. A mutant gene is isolated in which a transposon has inserted into the target gene and altered the resulting phenotype.

7.4.13.2.1 Isolation of the Mutated Gene

Using a probe for the transposon, the mutated gene can be isolated. Then, using the DNA adjacent to the transposon in the isolated, mutated gene as a probe, the normal wild-type allele of the target gene can be isolated. Such techniques are taught, for example, in McLaughlin and Walbot, Genetics, Vol. 117 pp. 771-776 (1987), as well as numerous other references.

7.4.13.2.2 Reporter Gene

In addition to the functional gene and the selectable marker gene, the DNA sequences may also contain a reporter gene which facilitates screening of the transformed shoots and plant material for the presence and expression of endogenous DNA sequences. Exemplary reporter genes include β -glucuronidase and luciferase.

7.4.13.2.3 Transfer Regions of a Suitable Plasmid

As described above, the exogenous DNA sequences are introduced into the area of the explants by incubation with Agrobacterium cells which carry the sequences to be transferred within a transfer DNA (T-DNA) region found on a suitable plasmid, typically the Ti plasmid. Ti plasmids contain two regions essential for the transformation of plant cells. One of these, the T-DNA region, is transferred to the plant nuclei and induces tumor formation. The other, referred to as the virulence (vir) region, is essential for the transfer of the T-DNA but is not itself transferred. By inserting the DNA sequence to be transferred into the T-DNA region, introduction of the DNA sequences to the plant genome can be effected. Usually, the Ti plasmid will be modified to delete or to inactivate the tumor-causing genes so that they are suitable for use as a vector for the transfer of the gene constructs of the present invention. Other plasmids may be utilized in conjunction with Agrobacterium for transferring the DNA sequences of the present invention to the plant cells.

The construction of recombinant Ti plasmids may be accomplished using conventional recombinant DNA techniques, such as those described by Sambrook et al. (1989). Frequently, the plasmids will include additional selective marker genes which permit manipulation and construction of the plasmids in suitable hosts, typically bacterial hosts other than Agrobacterium, such as *E. coli*. In addition to the above-described kanamycin resistance marker gene, other exemplary genes are the tetracycline resistance gene and the ampicillin resistance gene, among others.

7.4.13.2.4 Transcriptional and Translational Control Sequences

The genes within the DNA sequences will typically be linked to appropriate transcriptional and translational control sequences which are suitable for the *Pelargonium* plant host. For example, the gene will typically be situated at a distance from a promoter

corresponding to the distance at which the promoter is normally effective in order to ensure transcriptional activity. Usually, a polyadenylation site and transcription termination site will be provided at the 31-end of the gene coding sequence. Frequently, the necessary control functions can be obtained together with the structural gene when it is isolated from a target plant or other host. Such intact genes will usually include coding sequences, intron (s) , a promoter, enhancers, and all other regulatory elements either upstream (5') or downstream (3') of the coding sequences.

7.4.13.2.5 Using the Binary Vector System to Introduce the DNA Sequence

The binary vector system generally discussed above may be used to introduce the DNA sequence according to the present invention. A first plasmid vector strain would carry the T-DNA sequence while a second plasmid vector carries a virulence (vir) region. By incubating Agrobacterium cells carrying both plasmids with the explant, infection of the plant material is thus achieved.

7.4.13.2.6 The T-DNA Plasmid

Any one of a number of T-DNA plasmids can be used with such a binary vector system, the only requirement being that one be able to select independently for the two plasmids. The T-DNA plasmid in a preferred embodiment comprises a heterologous promoter which promotes the transcription of one or more genes within the exogenous DNA fragment (s) . An example is the Cauliflower Mosaic Virus 35S promoter (Odell et al., Nature, Vol. 313, pp. 810-812 (1985) among others.

7.4.13.2.7 Agrobacterium Species

Suitable Agrobacterium species include Agrobacterium tumefaciens and Agrobacterium rhizocienes. While the wild-type Agrobacterium rhizocrenes may be used, the Agrobacterium tumefaciens should be disarmed by deactivating its tumor activating capacity prior to use.

Preferred Agrobacterium tumefaciens strains include LBA4404, as described by Hoekema et al., Nature, Vol. 303, pp. 179-180 (1983) and EHA101 as described by Hood et al., J. Bacteriol., Vol. 168, pp. 1291-1301 (1986). A preferred Agrobacterium

rhizocrenes strain is 15834, as described by Birot et al., Plant Physiol. Biochem., Vol. 25, pp. 323-325 (1987).

7.4.13.2.8 Antisense Sequences

As the Agrobacterium strains carrying the desired exogenous DNA sequences are being prepared, the following antisense sequences are preferred for use in the present process, and are particularly preferred in transforming regal Pelargonium petiole explants according to the present method. These sequences are the known sequences for ACC Synthase (1-aminocyclopropane-1-carboxylate synthase) and ACC Oxidase (1-aminocyclopropane-1-carboxylate oxidase), reversed to create antisense sequences.

7.4.13.2.9 Culturing the Agrobacterium Strains

After the Agrobacterium strains carrying the desired exogenous DNA sequences have been prepared, they will usually be cultured for a period of time prior to incubation with the explant material. Initially, the Agrobacterium may be cultured on a solid media including nutrients, an energy source and a gelling agent. Suitable nutrients include salts, tryptone and yeast extracts, while most sugars are suitable as the energy source and the gelling agent can be TC agar or bactoagar or other similar products. The Agrobacterium cells are typically cultured for about two to five days, preferably in the dark at about 23-28° C, and are collected by scraping before browning (while still a white color). The cells are scraped from the medium and suspended in a liquid medium such as L-broth, pH 6.9-7.1, preferably 7.0. The bacteria are cultured in liquid medium for 8-36 hours, preferably 12-20, on a shaker (50-220 rpm, preferably 100-120 rpm) at 23-28° C. At the end of this period the bacteria are diluted to an optical density of 0.3 and cultured for 2-6, preferably 4, hours (on a shaker, 23-28° C). The Agrobacterium cells thus incubated are ready for inoculation onto explant material such as Pelargonium petiole sections.

When Agrobacterium cells are inoculated onto Pelargonium x domesticum petiole explants, and after the coincubation discussed above, the tissue culture method will proceed generally in accordance with the disclosure of U.S. Application Serial No. 08/149,702 filed November 9, 1993, which is a Continuation of U.S. Application Serial No. 07/690,073 filed April 23, 1991, by Wendy Oglevee- O'Donovan and Eleanor Stoots, both of which applications are hereby incorporated herein by reference. By means known



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7.4.14 TRANSFORMATION OF PLANT CELLS USING ALTERNATIVE METHODS

A variety of techniques are available for the introduction of the genetic material into or transformation of the plant cell host. However, the particular manner of introduction of the plant vector into the host is not critical to the practice of the present invention, and any method which provides for efficient transformation may be employed. In addition to transformation using plant transformation vectors derived from the tumor-inducing (Ti) or root-inducing (Ri) plasmids of Agrobacterium, alternative methods could be used to insert the DNA constructs of the present invention into plant cells. Such methods may include, for example, the use of liposomes, transformation using viruses or pollen, chemicals that increase the direct uptake of DNA (Paszowski et al., EMBO J., 3, 2717 (1984)), microinjection (Crossway et al., Mol. Gen. Genet., 202, 179 (1985)), electroporation (Fromm et al., Proc. Natl. Acad. Sci. US , 82, 824 (1985)), or high-velocity microprojectiles (Klein et al., Nature, 327, 70 (1987)).

7.4.14.1 Plant Tissue Source or Cultured Plant Cell

The choice of plant tissue source or cultured plant cells for transformation will depend on the nature of the host plant and the - transformation protocol. Useful tissue sources include callus, suspension culture cells, protoplasts, leaf segments, stem segments, tassels, pollen, embryos, hypocotyls, tuber segments, meristematic regions, and the like.

The tissue source is regenerable, in that it will retain the ability to regenerate whole, fertile plants following transformation.

7.4.14.2 Conditions During Transformation

The transformation is carried out under conditions directed to the plant tissue of choice. The plant cells or tissue are exposed to the DNA carrying the present multi-gene expression cassette for an effective period of time. This may range from a less-than-one-second pulse of electricity for electroporation, to a two-to-three day co-cultivation in the presence of plasmid-beazing Agrobacterium cells. Buffers and media used will also vary with the plant tissue source and transformation protocol. Many transformation protocols employ a feeder layer of suspended culture cells (tobacco or Black Mexican Sweet Corn,

7.4.15 METHOD FOR TRANSFORMATION OF THE TARGET PLANT

The methods used for the actual transformation of the target plant are not critical to this invention. The transformation of the plant is preferably permanent, e.g. by integration of introduced sequences into the plant genome, so that the introduced sequences are passed onto successive plant generations. There are many plant transformation techniques well-known to workers in the art, and new techniques are continually becoming known. Any technique that is suitable for the target plant can be employed with this invention. For example, the sequences can be introduced in a variety of forms, such as a strand of DNA, in a plasmid, or in an artificial chromosome, to name a few. The introduction of the sequences into the target plant cells can be accomplished by a variety of techniques, as well, such as calcium phosphate-DNA co-precipitation, electroporation, microinjection, Agrobacterium infection, liposomes or microprojectile transformation. Those of ordinary skill in the art can refer to the literature for details, and select suitable techniques without undue experimentation.

7.4.15.1 Introduction of Sequences into Target Plant Cells

It is possible to introduce the recombinase gene, in particular, into the transgenic plant in a number of ways. The gene can be introduced along with all of the other basic sequences, as in the first preferred embodiment described above. The repressible promoter/recombinase construct can be also introduced directly via a viral vector into a transgenic plant that contains the other sequence components of the system. Still another method of introducing all the necessary sequences into a single plant is the second preferred embodiment described above, involving a first transgenic plant containing the transiently-active promoter/structural gene sequences and the blocking sequence, and a second transgenic plant containing the recombinase gene linked to a germination-specific plant-active promoter, the two plants being hybridized by conventional to produce hybrid progeny containing all the necessary sequences.

It is also possible to introduce the recombinase itself directly into a transgenic plant as a conjugate with a compound such as biotin, that is transported into the cell. See Horn, et al. (1990).

7.4.15.2 Direct or Vectored Transformation

7.4.16 SUBCULTURING CELLS OR CALLUS GROWING IN NORMALLY INHIBITORY CONCENTRATIONS OF THE SELECTIVE AGENTS

Cells or callus observed to be growing in the presence of normally inhibitory concentrations of the selective agents are presumed to be transformed and may be subcultured several additional times on the same medium to remove non-resistant sections. The cells or calli can then be assayed for the presence of the viral gene cassette, or may be subjected to known plant regeneration protocols. In protocols involving the direct production of shoots, those shoots appearing on the selective media are presumed to be transformed and may be excised and rooted, either on selective medium suitable for the production of roots, or by simply dipping the excised shoot in a root-inducing compound and directly planting it in vermiculite.

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7.4.17 SELECTING FOR MULTI-VIRAL RESISTANCE

In order to produce transgenic plants exhibiting multi-viral resistance, the viral genes must be taken up into the plant cell and stably integrated within the plant genome. Plant cells and tissues selected for their resistance to an inhibitory agent are presumed to have acquired the selectable marker gene encoding this resistance during the transformation treatment.

Since the marker gene is commonly linked to the viral genes, it can be assumed that the viral genes have similarly been acquired. Southern blot hybridization analysis using a probe specific to the viral genes can then be used to confirm that the foreign genes have been taken up and integrated into the genome of the plant cell. This technique may also give some indication of the number of copies of the gene that have been incorporated. Successful transcription of the foreign gene into mRNA can likewise be assayed using Northern blot hybridization analysis of total cellular RNA and/or cellular RNA that has been enriched in a polyadenylated region. mRNA molecules encompassed within the scope of the invention are those which contain viral specific sequences derived from the viral genes present in the transformed vector which are of the same polarity to that of the viral genomic RNA such that they are capable of base pairing with viral specific RNA of the opposite polarity to that of viral genomic RNA under conditions described in Chapter 7 of Sambrook et al. (1989). mRNA molecules also encompassed within the scope of the invention are those which contain viral specific sequences derived from the viral genes present in the transformed vector which are of the opposite polarity to that of the viral genomic RNA such that they are capable of base pairing with viral genomic RNA under conditions described in Chapter 7 of Sambrook et al. (1989).

The presence of a viral gene can also be detected by immunological assays, such as the double-antibody sandwich assays described by Namba, et al., *Gene*, 107, 181 (1991) as modified by Clark et al., *J. Gen. Virol.*, 34, 475 (1979). See also, Namba et al., *Phytopathology*, 82, 940 (1992).

Virus resistance can be assayed via infectivity studies as generally disclosed by Namba et al., *ibid.*, wherein plants are scored as symptomatic when any inoculated leaf shows vein-clearing, mosaic or necrotic symptoms.

7.4.18 CELL CULTURE PRODUCTION OF MATURE HETEROLOGOUS PROTEIN

Transgenic cells, typically callus cells, are cultured under conditions that favor plant cell growth, until the cells reach a desired cell density, then under conditions that favor expression of the mature protein under the control of the given promoter. Preferred culture conditions are described herein. Purification of the mature protein secreted into the medium is by standard techniques known by those of skill in the art.

In one embodiment of the invention, in which BPN' is secreted as the proBPN' form of the enzyme, the chaperon "pro" moiety of the enzyme facilitates enzyme folding and is cleaved from the enzyme, leaving the active mature form of BPN'. In another embodiment, the mature enzyme is co-expressed and co-secreted with the "pro" chaperon moiety, with conversion of the enzyme to active form occurring in presence of the free chaperon (Eder et al., Biochem. (1993) L2:18-26; Eder et al, (1993) J. Mol. Biol. 223:293-304). In yet another embodiment of the invention, the BPN' is secreted in inactive form at a pH that may be in the 6-8 range, with subsequent activation of the inactive form, e.g., after enzyme isolation, by exposure to the "pro" chaperon moiety, e.g., immobilized to a solid support. In both of these embodiments, the culture medium is maintained at a pH of between 5 and 6, preferably about 5.5 during the period of active expression and secretion of BPN', to keep the BPN', which is normally active at alkaline pH, at a pH below optimal activity.

7.4.18.1 Production of Mature Heterologous Protein in Germinatin Seeds

In this embodiment, monocot cells transformed as above are used to regenerate plants, seeds from the plants are harvested and then germinated, and the mature protein is isolated from the germinated seeds.

Plant regeneration from cultured protoplasts or callus tissue is carried by standard methods, e.g., as described in Evans et al., HANDBOOK OF PLANT CELL CULTURE Vol. 1: (MacMillan Publishing Co. New York, 1983); and Vasil I.R. (ed.), CELL CULTURE AND SOMATIC CELL GENETICS OF PLANTS, Acad. Press, Orlando, Vol. 1, 1984, and Vol. 111, 1986, and as described in the above-cited PCT application.

7.4.19 PRODUCTION OF MATURE HETEROLOGOUS PROTEIN IN MATURING SEEDS

In this embodiment, monocot cells transformed as above are used to regenerate plants, and seeds from the plants are allowed to mature, typically in the field, with consequent production of heterologous protein in the seeds.

Alternatively, the seeds may be fractionated by standard methods to obtain the heterologous protein in enriched or purified form. In one general approach, the seed is first milled, then suspended in a suitable extraction medium, e.g., an aqueous or an organic solvent, to extract the protein or metabolite of interest. If desired the heterologous protein can be further fractionated and purified, using standard purification methods.

The following examples are provided by way of illustration only and not by way of limitation. Those of skill will readily recognize a variety of noncritical parameters which could be changed or modified to yield essentially similar results.

7.4.20 REGENERATION OF THE TRANSFORMED PLANT CELLS

After transformation, the transformed plant cells must be regenerated.

The methods used to regenerate transformed cells into whole plants are not critical to this invention, and any method suitable for the target plant can be employed. The literature describes numerous techniques for regenerating specific plant types, (e.g., via somatic embryogenesis, Umbeck, et al., 1987) and more are continually becoming known. Those of ordinary skill in the art can refer to the literature for details and select suitable techniques without undue experimentation.

Plant regeneration from cultured protoplasts is described in Evans et al., Handbook of Plant Cell Cultures, Vol. 1: (MacMillan Publishing Co., New York, 1983); and Vasil I.R. (ed.), Cell Culture and Somatic Cell Genetics of Plants, Acad. Press, Orlando, Vol. I, 1984, and Vol.-III (1986), which are hereby incorporated is by reference.

It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to, all major species of sugarcane, sugar beets, cotton, fruit trees, and legumes.

Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing explants is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced in the callus tissue. These embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins.. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is usually reproducible and repeatable.

7.4.21 BREEDING TECHNIQUES

After the expression cassette is stably incorporated in transgenic plants, it can be transferred to other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

Seed from plants regenerated from tissue culture is grown in the field and self-pollinated to generate true breeding plants. The progeny from these plants become true breeding lines which are evaluated for viral resistance in the field under a range of environmental conditions. The commercial value of viral-resistant plants is greatest if many different hybrid combinations with resistance are available for sale. The farmer typically grows more than one kind of hybrid based on such differences as maturity, disease and insect resistance, color or other agronomic traits. Additionally, hybrids adapted to one part of a country are not adapted to another part because of differences in such traits as maturity, disease and insect tolerance, or public demand for specific varieties in given geographic locations.

Because of this, it is necessary to breed viral resistance into a large number of parental lines so that many hybrid combinations can be produced.

Adding viral resistance to agronomically elite lines is most efficiently accomplished when the genetic control of viral resistance is understood. This requires crossing resistant and sensitive plants and studying the pattern of inheritance in segregating generations to ascertain whether the trait is expressed as dominant or recessive, the number of genes involved, and any possible interaction between genes if more than one are required for expression. With respect to transgenic plants of the type disclosed herein, the transgenes exhibit dominant, single gene Mendelian behavior. This genetic analysis can be part of the initial efforts to covert agronomically elite, yet sensitive lines to resistant lines. A conversion process (backcrossing) is carried out by crossing the original resistant line with a sensitive elite line and crossing the progeny back to the sensitive parent. The progeny from this cross will segregate such that some plants carry the resistance gene(s) whereas some do not. Plants carrying the resistance gene(s) will be crossed again to the sensitive parent resulting in progeny which segregate for resistance and sensitivity once more. This is repeated until the original sensitive parent has been

Suitable plants include dicots and monocots. More particularly, useful crop plants can include: alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, turnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, papaya, and sugarcane.

Examples of suitable ornamental plants are: *Arabidopsis thaliana*, *Saintpaulia*, *petunia*, *pelargonium*, *poinsettia*, *chrysanthemum*, *carnation*, and *zinnia*.

The plants used in the process of the present invention are derived from monocots, particularly the members of the taxonomic family known as the Gramineae. This family includes all members of the grass family of which the edible varieties are known as cereals. The cereals include a wide variety of species such as wheat (*Triticwn* sps.), rice (*Oryza* sps.) barley (*Hordewn* sps.) oats, (*Avena* sps.) rye (*Secale* sps.), corn (*Zea* sps.) and millet (*Pennisettum* sps.). In the present invention, preferred family members are rice and barley.

7.4.22 IDENTIFICATION AND LOCALIZATION AND INTROGRESSION INTO PLANTS OF DESIRED MULTIGENIC TRAITS WITH RFLP TECHNOLOGY

The invention typically involves genetic linkage maps constructed with RFLP technology and the use of RFLP probes to correlate those probes with Quantitative Trait Loci (QTL) and the degree of inheritance of particular multigenic traits.

7.4.22.1 Determining the Degree of Inheritance of Particular Multigenic Traits

In one embodiment, a plant source (designated P_1) having a desired multigenic trait--for example, increased height--is recovered and crossed with a second plant (designated P_2) having essentially or substantially opposite characteristics, that is, decreased height. Heterozygote plants from the F_1 population are selfed to create a segregating (F_2) plant population which exhibit a gradient with respect to height, i.e., with respect to the degree of expression of the multigenic or quantitative trait of interest.

7.4.22.1.1 RFLP Probe Tests

Quantitative values for the trait of interest (height) are determined and assigned to each individual parent plant, F_1 population plant, and F_2 segregation plant and a genomic DNA sample from each plant is prepared for Southern blotting. Following preparation for a Southern blot--which may be constructed to contain, for example, DNA from 25 to 50 or more different F_2 plants--an RFLP probe is randomly chosen or selected from an RFLP genetic linkage map and hybridized to create the blot. Additional Southern blots are constructed using other RFLP probes. As indicated above, the RFLPs to be used for this purpose, i.e., the indirect selection of a QTL, may but need not be randomly chosen. They can be selected in systematic fashion from the RFLP genetic linkage map. For example, for a trait of completely unknown location, several spaced RFLPs from each of the 10 chromosomes of maize may be selected for Southern blot testing for the location of DNA associated with a desired height. Alternatively, of course, all mapped RFLPs may be used.

Following these Southern blot constructs, a matrix may be prepared having an identification of each plant that has been tested, followed by its quantitative trait measurement (height) and the genotype as revealed by each RFLP probe tested. Typically, only three genotypes will be seen: P_1 , P_2 and F_1 , the latter being heterozygous and having one chromosome from each parent. Thus, from the matrix all plants can be grouped into

one of three RFLP genotypic categories: P_1P_1 , P_1P_2 or P_2P_2 . If, with one or more RFLPs, the so-grouped plants, when averaged, all show approximately equal expression of the trait of interest, i.e., the average height of plants in all groups is about the same, that RFLP is deemed noninformative. In other words, there was no association between the trait of interest and that particular RFLP or RFLPs. The genotype of the plant at the location of the RFLP was not relevant to the trait of interest.

7.4.22.1.2 Alteration of Experiment

Another RFLP, however, may show association with height. For example, the average height of the maize plants, respectively, in each of the P_1P_1 , P_1P_2 and P_2P_2 groups for a different RFLP, respectively, may be 3 feet, 4 feet and 5 feet. With this information, it may be presumed that this RFLP, as revealed by the degree of its correlation to the P_2P_2 genotype, hybridizes to maize DNA in the area of a gene for height.

7.4.22.1.3 Use of a Genetic Linkage Map

In the above-described manner, it is possible to review results from a first group of RFLP probes used to screen for association to the trait of interest. Use of an RFLP genetic linkage map allows the selection of further RFLPs to be tested on an objective, rather than random, basis. Correlation may be improved by testing RFLPs located on either side of that RFLP or RFLPs which initially showed the strongest association. Once the best probe or probes are identified they may then be utilized, by way of example, in a breeding program to select plants having a desired height.

It is to be noted, of course, that in a multigenic system, there may be three, four, or more different genes contributing to one trait. In such a situation there may, therefore, be many different quantitative expressions of that trait and no one gene can account for, or be relied upon to predict, that expression. We have further determined that the relative importance of each correlating RFLP can be determined. Particular values can be assigned to those RFLPs and utilized in a mathematical model to assist in predicting the degree of trait expression in a particular plant.

7.4.22.1.4 Analysis of Variance

In the case of RFLP analysis, there are three different genotypic classes, i.e., AA, Aa and aa. Among three classes there are two possible contrasts and, hence, there are 2 degrees of freedom among genotypic classes. Moreover, the two possible contrasts among genotypes can be specifically partitioned into linear and quadratic sources, each with one degree of freedom. The linear contrast would have the values -1, 0 and +1 for the three genotypic classes AA, Aa and aa, respectively. This effectively compares the difference between the AA class and the aa class, i.e., the parental classes. The values for the quadratic contrast are 1, -2 and 1 for the three genotypic classes and, therefore, compare the difference between the mean of the parental AA and aa classes to the heterozygote class Aa.

The error, or deviation from regression, reflects the failure of the observed points to be exactly on the regression line. In the case of RFLP "A" we can observe that the mean square for the linear contrast is approximately 100 times larger than that of the quadratic contrast and about 10 times larger than the error mean square. The magnitudes of the mean squares from the analysis of variance indicates that linear regression can account for the majority of the variance among the plant heights. The r^2 (coefficient of determination) value can be defined as the sums of the linear and quadratic mean squares divided by the total phenotypic variance (the total sums of squares of deviations from mean). The r^2 value for "A" indicates that 89% of the observed variance for plant height can be explained as due to differences among RFLP "A" genotypic classes. Similarly, the analysis of variance for RFLP "B" indicates that none of the variance for plant height can be explained due to differences among genotypic classes. The analysis of variance for RFLP "C" indicates that 53% of the variance can be explained due to differences among genotypic classes and that both the linear and quadratic contrast are important. Thus, the relative magnitudes of mean squares from the analysis of variance provides a description of the relationship between RFLPs and plant height. The conclusions coincide with those obtained from observation of the plots of plant height by genotypic classes.

The means for each genotypic class are also presented in Table 2. These means represent the average phenotypic performance for each of the 3 genotypic classes. For RFLP "A", the mean of the parent 1 class (aa) is 12.5, the mean for the heterozygous class (Aa) is 62.5, and the mean for the homozygous parent 2 class (AA) is 100 plant height

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units. The linear regression line slope (b_1) is also presented for each RFLP. Thus, the linear regression of plant height on genotypic classes for RFLP "A" is 45. This, as noted above, can be interpreted as indicating that each time an "A" allele is substituted for an a allele that plant height will increase 45 units. The slope thus permits comparisons among RFLPs and helps to identify those RFLPs most strongly associated with the expression of a trait of interest.

Once the RFLPs most strongly associated with the expression of a trait are identified, the effects of each RFLP may be combined into a multiple regression model which will permit prediction of expression of the trait of interest based on knowledge of the genotypes of specific RFLP. The effects of each RFLP are not strictly additive because the effects of RFLP may be correlated. For example, in this case, RFLP "A" and "C" may each provide some unique information, but part of the information provided by one RFLP may also be provided by another. Thus, the average effects of allelic substitutions may not be simply added together to provide a predictive model.

7.4.22.2 General Form of Multiple Regression Model

The general form of a useful multiple regression predictive model is shown below:

$$y = \mu + b_1 (\text{genotype of RFLP locus 1}) + b_2 (\text{genotype of RFLP locus 2})$$

where y is the predicted expression of a trait, μ is the weighted mean expression of the trait for the population, b_1 is the coefficient associated with a specific RFLP and so on. The genotype of a RFLP will be -1, 0 or 1 for genotypic classes AA, Aa and aa if the RFLP is additive (linear), or 1, -2 or 1 if the RFLP is quadratic (non-additive).

Determination of the "best" multiple regression model requires an iterative process of substitution of RFLPs into and out of the model and the evaluation of interaction (epistatic) effects of RFLPs. For example, if two independent genes act together to provide expression of a trait, the genotypic class may involve the products of linear and/or quadratic genotypic values. The process for determination of the "best" multiple regression model can be done using stepwise regression procedures or by comparison of partial and sequential sums of squares from the regression models.

7.1.4.22.2.1 Example of Model Building

For example, in the present hypothetical, the logical RFLPs to include in the model would be the linear contrast for RFLP "A" and both the linear and quadratic contrasts for RFLP "C". The partial and sequential sums of squares (SS) for a model including RFLP A and C are the following:

Source	sequential SS	partial SS
linear "A"	5580	2812
linear "C"	62	236
quadratic "C"	503	503

The magnitudes of the sums of squares indicates that linear "A" is the most important determinate in explaining the variance for plant height. However, the reduction in partial SS (2812) vs. sequential SS (5580) for linear "A" suggests that much of the effect of linear "A" is accounted for by the linear and quadratic effects of "C". It should be noted that if the effects of each locus were completely independent there would be no differences in the partial and sequential sums of squares. Changes in the magnitudes of these sums of squares in an analysis of variance indicates that the effects of the different RFLP are correlated.

7.4.22.2.2 Another Model Constructed (Eliminate Linear Contrast)

The iterative process of substituting linear and quadratic contrasts for different RFLPs can continue until a final predictive model is constructed. The objective of the model is to maximize the r^2 (coefficient of determination) value using as few RFLP as possible as predictive variables. The final model will generally eliminate RFLP which might flank a particular gene of interest because both RFLP will typically be contributing the same information. In addition, the final model might contain effects with reflect interactions between RFLPs.

In the present hypothetical, the next step in the process of model building might be to eliminate the linear contrast for RFLP "C". The results of this model are the following:

Source	sequential SS	partial SS
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linear "A"	5580	5127
quadratic "C"	328	328

7.4.22.3 Final Mathematical Model

The general agreement in magnitude between partial and sequential SS suggest that the effects of linear "A" and quadratic "C" are relatively independent. The r^2 value for this model is 0.95. Thus, 95% of the variance for plant height can be explained as due to differences among genotypic classes at RFLP "A" and "C". The final prediction equation would, therefore, be written as follows:

$$\text{Plant height} = 61.4 + 43 (\text{linear code RFLP A}) - 7.0 (\text{quadratic code for RFLP C}).$$

Thus, if the genotypes of RFLP A and C are known for a particular plant, the height of that plant can be predicted without having to measure its height following growth to maturity. In a breeding program, the breeder can analyze the genotypes of specific RFLP of seedling plants grown in a greenhouse during the winter and need only evaluate those plants predicted to have the desired plant height in the field the following summer.

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7.4.22.3.1 Explanation of the Mathematical Model

TABLE 6								
Hypothetical Raw data								
Genotype Observed	"A" (Linear Code)		"B" (Linear Code)		"C" (Linear Code)		(Quadratic Code)	Plant Height
1	AA	1	bb	-1	CC	1	1	100
2	aa	-1	bb	-1	cc	-1	1	0
3	Aa	0	BB	1	C	c	0	-275
4	Aa	0	Bb	0	CC	1	1	50
5	aa	-1	BB	1	CC	1	1	25

TABLE 7				
Analysis of Variance				
Source of Variance	Degrees of Freedom	RFLP locus		
		A	B	C
		Mean Squares		
Genotype	2			
Linear	1	5104	0.0	2552
Quadratic	1	44	0.0	1575
Error (deviations from regression)	2	625	6250	1458
r ²		0.89	0.0	0.53
Genotypes	(code)	Means		
Homozygous Parent 1	(-1)	12.5	50	0
Heterozygote	(0)	62.5	50	75
Homozygous Parent 2	(+1)	100.0	50	58
Slope(b1)		45	0	23

expression of a quantitative trait are difficult to identify and Mendelian analysis cannot be applied. Id. Quantitative genetic analysis has therefore focused on estimation of breeding value which is the sum of the effects of the alleles at many loci. Nevertheless, a basic premise of quantitative genetics is that the laws which govern the inheritance of quantitative loci are the same as those which govern qualitative loci. The magnitude of the individual allelic effects which can be resolved through RFLP analysis will be a function of experimental error and recombination frequencies. As RFLP maps are developed which more completely saturate the genome, identification of more loci with smaller individual effects should be possible. Thus, RFLP analysis also offers the opportunity to determine the effects of individual loci (alleles) with major effects, and thereby to reduce the analysis of complex quantitative traits to classical Mendelian segregation ratios of individual alleles.

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8. MUTAGENIZING ORGANISMS - ENGINEERING ASPECTS

8.1.1 GENERAL CONSIDERATIONS

Cells have a number of well-established uses in molecular biology. For example, cells are commonly used as hosts for manipulating DNA in processes such as transformation and recombination. Cells are also used for expression of recombinant proteins encoded by DNA transformed into the cells. Some types of cells are also used as progenitors for generation of transgenic animals and plants. Although all of these processes are now routine, in general, the genomes of the cells used in these processes have evolved little from the genomes of natural cells, and particularly not toward acquisition of new or improved properties for use in the above processes.

Probably, most genes do have cooperative functions. Finally, this approach can only explore a very limited number of the total number of permutations even for a single gene. For example, varying even ten positions in a protein with every possible amino acid would generate 20^{10} variants, which is more than can be accommodated by existing methods of transfection and screening.

In view of these limitations, the traditional approach is inadequate for improving

cellular genomes in many useful properties. For example, to improve a cell's capacity to express a recombinant protein might require modification in any or all of a substantial number of genes, known and unknown, having roles in transcription, translation, posttranslational modification, secretion or proteolytic degradation, among others. Attempting individually to optimize even all the known genes having such functions would be a virtually impossible task, let alone optimizing hitherto unknown genes which may contribute to expression in manners not yet understood.

The present invention provides inter alia novel methods for evolving the genome of whole cells and organisms which overcome the difficulties and limitations of prior methods.

This ability to evolve genes artificially is of fundamental importance. For example, cells have a number of well-established uses in molecular biology, medicine and industrial processes. For example, cells are commonly used as hosts for manipulating DNA in processes such as transformation and recombination. Cells are used for expression of recombinant proteins encoded by DNA transformed/transfected or otherwise introduced into the cells. Some types of cells are used as progenitors for generation of transgenic animals and plants. The genomes of the cells used in these processes had evolved little from the genomes of natural cells, and particularly not toward acquisition of new or improved properties for use in the above processes.

Additional methods of recursively recombining nucleic acids in vivo and selecting resulting recombinants would be of use. The present invention provides a number of new and valuable methods and compositions for whole and partial genome evolution.

Metabolic engineering is the manipulation of intermediary metabolism through the use of both classical genetics and genetic engineering techniques. Cellular engineering is generally a more inclusive term referring to the modification of cellular properties. Cameron et al. (Applied Biochem. Biotech. 38:105-140 (1993)) provide a summary of equivalent terms to describe this type of engineering, including "metabolic engineering",

which is most often used in the context of industrial microbiology and bioprocess engineering, "in vitro evolution" or "directed evolution", most often used in the context of environmental microbiology, "molecular breeding", most often used by Japanese researchers, "cellular engineering", which is used to describe modifications of bacteria, animal, and plant cells, "rational strain development", and "metabolic pathway evolution". In this application, the terms "metabolic engineering" and "cellular engineering" are used preferentially for clarity; the term "evolved" genes is used as discussed below.

Metabolic engineering can be divided into two basic categories: modification of genes endogenous to the host organism to alter metabolite flux and introduction of foreign genes into an organism. Such introduction can create new metabolic pathways leading to modified cell properties including but not limited to synthesis of known compounds not normally made by the host cell, production of novel compounds (e.g. polymers, antibiotics, etc.) and the ability to utilize new nutrient sources. Specific applications of metabolic engineering can include the production of specialty and novel chemicals, including antibiotics, extension of the range of substrates used for growth and product formation, the production of new catabolic activities in an organism for toxic chemical degradation, and modification of cell properties such as resistance to salt and other environmental factors.

Bailey (Science 252:1668-1674 (1991)) describes the application of metabolic engineering to the recruitment of heterologous genes for the improvement of a strain, with the caveat that such introduction can result in new compounds that may subsequently undergo further reactions, or that expression of a heterologous protein can result in proteolysis, improper folding, improper modification, or unsuitable intracellular location of the protein, or lack of access to required substrates. Bailey recommends careful configuration of a desired genetic change with minimal perturbation of the host. Liao (Curr. Opin. Biotech. 4:211-216 (1993)) reviews mathematical modeling and analysis of metabolic pathways, pointing out that in many cases the kinetic parameters of enzymes are unavailable or inaccurate.

Stephanopoulos et al. (Trends. Biotechnol. 11:392-396 (1993)) describe attempts to improve productivity of cellular systems or effect radical alteration of the flux through

primary metabolic pathways as having difficulty in that control architectures at key branch points have evolved to resist flux changes. They conclude that identification and characterization of these metabolic nodes is a prerequisite to rational metabolic engineering. Similarly, Stephanopoulos (Curr. Opin. Biotech. 5:196-200 (1994)) concludes that rather than modifying the "rate limiting step" in metabolic engineering, it is necessary to systematically elucidate the control architecture of bioreaction networks. The present invention is generally directed to the evolution of new metabolic pathways and the enhancement of bioprocessing through a process herein termed recursive sequence recombination. Recursive sequence recombination entails performing iterative cycles of recombination and screening or selection to "evolve" individual genes, whole plasmids or viruses, multigene clusters, or even whole genomes (Stemmer, Bio/Technology 13:549-553 (1995)). Such techniques do not require the extensive analysis and computation required by conventional methods for metabolic engineering. Recursive sequence recombination allows the recombination of large numbers of mutations in a minimum number of selection cycles, in contrast to traditional, pair wise recombination events.

Thus, because metabolic and cellular engineering can pose the particular problem of the interaction of many gene products and regulatory mechanisms, recursive sequence recombination (RSR) techniques provide particular advantages in that they provide recombination between mutations in any or all of these, thereby providing a very fast way of exploring the manner in which different combinations of mutations can affect a desired result, whether that result is increased yield of a metabolite, altered catalytic activity or substrate specificity of an enzyme or an entire metabolic pathway, or altered response of a cell to its environment.

8.1.2 THE EVOLUTIONARY IMPORTANCE OF RECOMBINATION

Strain improvement is the directed evolution of an organism to be more "fit" for a desired task. In nature, adaptation is facilitated by sexual recombination. Sexual recombination allows a population to exploit the genetic diversity within it, e.g., by consolidating useful mutations and discarding deleterious ones. In this way, adaptation and evolution can proceed in leaps. In the absence of a sexual cycle, members of a population

8.1.2.1 DNA STOCHASTIC &/OR NON-STOCHASTIC MUTAGENESIS VS NATURAL RECOMBINATION

DNA stochastic &/or non-stochastic mutagenesis includes the recursive recombination of DNA sequences. A significant difference between DNA stochastic &/or non-stochastic mutagenesis and natural sexual recombination is that DNA stochastic &/or non-stochastic mutagenesis can produce DNA sequences originating from multiple parental sequences while sexual recombination produces DNA sequences originating from only two parental sequences.

In sequential random mutagenesis, useful mutations are accumulated one per selection event. Many useful mutations are discarded each cycle in favor of the best performer, and neutral or deleterious mutations which survive are as difficult to lose as they were to gain and thus accumulate. In sexual evolution pairwise recombination allows mutations from two different parents to segregate and recombine in different combinations. Useful mutations can accumulate and deleterious mutations can be lost. Poolwise recombination, such as that effected by DNA stochastic &/or non-stochastic mutagenesis, has the same advantages as pairwise recombination but allows mutations from many parents to consolidate into a single progeny. Thus poolwise recombination provides a means for increasing the number of useful mutations that can accumulate each selection event. One can plot the potential number of mutations an individual can accumulate by each of these processes. Recombination is exponentially superior to sequential random mutagenesis, and this advantage increases exponentially with the number of parents that can recombine. Sexual recombination is thus more conservative. In nature, the pairwise nature of sexual recombination may provide important stability within a population by impeding the large changes in DNA sequence that can result from poolwise recombination. For the purposes of directed evolution, however, poolwise recombination is more efficient.

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sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps.

Another example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., J Mol. Biol. 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al, supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative- scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W , T , and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a word length (W) of 11, an expectation (E) of 10, $M=5$, $N=-4$, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word length (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)).

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin &

Altschul, Proc. Natl. Acad. Sci. USA 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability ($P(N)$), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0. 1, more preferably less than about 0. 0 1, and most preferably less than about 0.001.

A further indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions.

Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions.

The term "**naturally-occurring**" is used to describe an object that can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally- occurring. Generally, the term naturally-occurring refers to an object as present in a non- pathological (undiseased) individual, such as would be typical for the species.

Asexual recombination is recombination occurring without the fusion of gametes to form a zygote.

A "**mismatch repair deficient strain**" can include any mutants in any organism impaired in the functions of mismatch repair. These include mutant gene products of mutS, mutT, mutH, mutL, ovrD, dcm, vsr, umuC, umuD, sbcB, recJ, etc. The impairment is achieved by genetic mutation, allelic replacement, selective inhibition by an added reagent such as a small compound or an expressed antisense RNA, or other techniques. Impairment can be of the genes noted, or of homologous genes in any organism.

[illegible][illegible][illegible][illegible]

Table 1. Mean values of the variables measured during the three trials

[illegible][illegible][illegible][illegible][illegible][illegible][illegible]

8.2.1.3.3 PERFORM IN VIVO RECOMBINATION

The invention further provides methods for performing in vivo recombination. At least first and second segments from at least one gene are introduced into a cell, the segments differing from each other in at least two nucleotides, whereby the segments recombine to produce a library of chimeric genes. A chimeric gene is selected from the library having acquired a desired function.

The invention further provides methods of evolving a cell to acquire a desired function. These methods entail providing a population of different cells. The cells are cultured under conditions whereby DNA is exchanged between cells, forming cells with hybrid genomes. The cells are then screened or selected for cells that have evolved toward acquisition of a desired property. The DNA exchange and screening/selecting steps are repeated, as needed, with the screened/selected cells from one cycle forming the population of different cells in the next cycle, until a cell has acquired the desired property.

Mechanisms of DNA exchange include conjugation, phage-mediated transduction, liposome delivery, protoplast fusion, and sexual recombination of the cells. Optionally, a library of DNA fragments can be transformed or electroporated into the cells.

8.2.1.3.4 PROTOPLAST-MEDIATED EXCHANGE

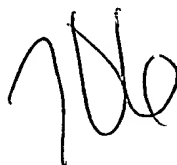
As noted, some methods of evolving a cell to acquire a desired property are effected by protoplast-mediated exchange of DNA between cells. Such methods entail forming protoplasts of a population of different cells. The protoplasts are then fused to form hybrid protoplasts, in which genomes from the protoplasts recombine to form hybrid genomes. The hybrid protoplasts are incubated under conditions promoting regeneration of cells. The regenerated cells can be recombined one or more times (i.e., via protoplasting or any other method than combines genomes of cells) to increase the diversity of any resulting cells. Preferably, regenerated cells are recombined several times, e.g., by protoplast fusion to generate a diverse population of cells. The next step is to select or screen to isolate regenerated cells that have evolved toward acquisition of the desired

property. DNA exchange and selection/screening steps are repeated, as needed, with regenerated cells in one cycle being used to form protoplasts in the next cycle until the regenerated cells have acquired the desired property. Industrial microorganisms are a preferred class of organisms for conducting the above methods. Some methods further comprise a step of selecting or screening for fused protoplasts free from unfused protoplasts of parental cells. Some methods further comprise a step of selecting or screening for fused protoplasts with hybrid genomes free from cells with parental genomes. In some methods, protoplasts are provided by treating individual cells, mycelia or spores with an enzyme that degrades cell walls. In some methods, the strain is a mutant that is lacking capacity for intact cell wall synthesis, and protoplasts form spontaneously. In some methods, protoplasts are formed by treating growing cells with an inhibitor of cell wall formation to generate protoplasts. In some methods, the desired property is expression and/or secretion of a protein or secondary metabolite, such as an industrial enzyme, a therapeutic protein, a primary metabolite such as lactic acid or ethanol, or a secondary metabolite such as erythromycin cyclosporin A or taxol. In other methods it is the ability of the cell to convert compounds provided to the cell to different compounds. In yet other methods, the desired property is capacity for meiosis. In some methods, the desired property is compatibility to form a heterokaryon with another strain.

The invention further provides methods of evolving a cell toward acquisition of a desired property. These methods entail providing a population of different cells. DNA is isolated from a first subpopulation of the different cells and encapsulated in liposomes. Protoplasts are formed from a second subpopulation of the different cells. Liposomes are fused with the protoplasts, whereby DNA from the liposomes is taken up by the protoplasts and recombines with the genomes of the protoplasts. The protoplasts are incubated under regenerating conditions. Regenerating or regenerated cells are then selected or screened for evolution toward the desired property.

8.2.1.3.4 REITERATIVE POOLING AND BREEDING OF HIGHER ORGANISMS

The method also provides methods of reiterative pooling and breeding of higher organisms. In the methods, a library of diverse multicellular organisms are produced (e.g.,



plants, animals or the like). A pool of male gametes is provided along with a pool of female gametes. At least one of the male pool or the female pool comprises a plurality of different gametes derived from different strains of a species or different species. The male gametes are used to fertilize the female gametes. At least a portion of the resulting fertilized gametes grow into reproductively viable organisms. These reproductively viable organisms are crossed (e.g., by pairwise pooling and joining of the male and female gametes as before) to produce a library of diverse organisms. The library is then selected for a desired trait or property.

The library of diverse organisms can comprise a plurality of plants such as Gramineae, Fetucoideae, Poacoideae, Agrostis, Phleum, Dactylis, Sorgum, Setaria, Zea, Oryza, Triticurn, Secale, Avena, Hordeurn, Saccharum, Poa, Festuca, Stenotaphrum, Cynodon, Coix, Olyreae, Phareae, Compositae or Leguminosae. For example, the plants can be e.g., corn, rice, wheat, rye, oats, barley, pea, beans, lentil, peanut, yam bean, cowpeas, velvet beans, soybean, clover, alfalfa, lupine, vetch, lotus, sweet clover, wisteria, sweet pea, sorghum, millet, sunflower, canola or the like.

Similarly, the library of diverse organisms can include a plurality of animals such as non-human mammals, fish, insects, or the like.

Optionally, a plurality of selected library members can be crossed by pooling gametes from the selected members and repeatedly crossing any resulting additional reproductively viable organisms to produce a second library of diverse organisms (e.g., by split pair wise pooling and rejoining of the male and female gametes). Here again, the second library can be selected for a desired trait or property, with the resulting selected members forming the basis for additional poolwise breeding and selection. A feature of the invention is the libraries made by these (or any preceding) method.

8.3. ORIGIN OF CELLS

8.3.1 EMBRYONIC CELLS OF AN ANIMAL

In some methods, the plurality of cells are embryonic cells of an animal, and the method further comprises propagating the transformed cells to transgenic animals. The



plurality of cells can be a plurality of industrial microorganisms that are enriched for microorganisms which are tolerant to desired process conditions (heat, light, radiation, selected pFL presence of detergents or other denaturants, presence of alcohols or other organic molecules, etc.).

8.3.2 ARTIFICIAL CHROMOSOMES

The invention further provides methods of evolving a cell toward acquisition of a desired property using artificial chromosomes. Such methods entail introducing a DNA fragment library cloned into an artificial chromosome into a population of cells. The cells are then cultured under conditions whereby sexual recombination occurs between the cells, and DNA fragments cloned into the artificial chromosome recombines by homologous recombination with corresponding segments of endogenous chromosomes of the populations of cells, and endogenous chromosomes recombine with each other. Cells can also be recombined via conjugation. Any resulting cells can be recombined via any method noted herein, as many times as desired, to generate a desired level of diversity in the resulting recombinant cells. In any case, after generating a diverse library of cells, the cells that have evolved toward acquisition of the desired property are screened and/or selected for a desired property. The method is then repeated with cells that have evolved toward the desired property in one cycle forming the population of different cells in the next cycle. Here again, multiple cycles of in vivo recombination are optionally performed prior to any additional selection or screening steps.

The invention further provides methods of evolving a DNA segment cloned into an artificial chromosome for acquisition of a desired property. These methods entail providing a library of variants of the segment, each variant cloned into separate copies of an artificial chromosome. The copies of the artificial chromosome are introduced into a population of cells. The cells are cultured under conditions whereby sexual recombination occurs between cells and homologous recombination occurs between copies of the artificial chromosome bearing the variants. Variants are then screened or selected for evolution toward acquisition of the desired property.

The invention further provides hyperrecombinogenic recA proteins.

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(a) recombining at least a first and second DNA segment from at least one gene conferring ability to catalyze a reaction of interest, the segments differing from each other in at least two nucleotides, to produce a library of recombinant genes;

(b) screening at least one recombinant gene from the library that confers enhanced ability to catalyze the reaction of interest by the cell relative to a wild type form of the gene;

(c) recombining at least a segment from at least one recombinant gene with a further DNA segment from at least one gene, the same or different from the first and second segments, to produce a further library of recombinant genes;

(d) screening at least one further recombinant gene from the further library of recombinant genes that confers enhanced ability to catalyze the reaction of interest in the cell relative to a previous recombinant gene;

(e) repeating (c) and (d), as necessary, until the further recombinant gene confers a desired level of enhanced ability to catalyze the reaction of interest by the cell.

Another aspect of the invention is a method of evolving a gene to confer ability to catalyze a reaction of interest, the method comprising:

(1) recombining at least first and second DNA segments from at least one gene conferring ability to catalyze a reaction of interest, the segments differing from each other in at least two nucleotides, to produce a library of recombinant genes;

(2) screening at least one recombinant gene from the library that confers enhanced

ability to catalyze a reaction of interest relative to a wild type form of the gene;

(3) recombining at least a segment from the at least one recombinant gene with a further DNA segment from the at least one gene, the same or different from the first and second segments, to produce a further library of recombinant genes;

(4) screening at least one further recombinant gene from the further library of recombinant genes that confers enhanced ability to catalyze a reaction of interest relative to a previous recombinant gene;

(5) repeating (3) and (4), as necessary, until the further recombinant gene confers a desired level of enhanced ability to catalyze a reaction of interest.

8.4.2. METHOD TO GENERATE A NEW BIOCATALYTIC ACTIVITY IN A CELL

A further aspect of the invention is a method of generating a new biocatalytic activity in a cell, comprising:

(1) recombining at least first and second DNA segments from at least one gene conferring ability to catalyze a first reaction related to a second reaction of interest, the segments differing from each other in at least two nucleotides, to produce a library of recombinant genes;

(2) screening at least one recombinant gene from the library that confers a new ability to catalyze the second reaction of interest;

(3) recombining at least a segment from at least one recombinant gene with a further DNA segment from the at least one gene, the same or different from the first and second segments, to produce a further library of recombinant genes;

(4) screening at least one further recombinant gene from the further library of recombinant genes that confers enhanced ability to catalyze the second reaction of interest in the cell relative to a previous recombinant gene;

(5) repeating (3) and (4), as necessary, until the further recombinant gene confers a desired level of enhanced ability to catalyze the second reaction of interest in the cell.

8.4.3. METHOD TO MODIFY A METABOLIC PATHWAY EVOLVED BY RECURSIVE SEQUENCE RECOMBINATION

Another aspect of the invention is a modified form of a cell, wherein the modification comprises a metabolic pathway evolved by recursive sequence recombination.

A further aspect of the invention is a method of optimizing expression of a gene product, the method comprising:

(1) recombining at least first and second DNA segments from at least one gene conferring ability to produce the gene product, the segments differing from each other in at least two nucleotides, to produce a library of recombinant genes;

(2) screening at least one recombinant gene from the library that confers optimized expression of the gene product relative to a wild type form of the gene;

(3) recombining at least a segment from the at least one recombinant gene with a further DNA segment from the at least one gene, the same or different from the first and second segments, to produce a further library of recombinant genes;

(4) screening at least one further recombinant gene from the further library of recombinant genes that confers optimized ability to produce the gene product relative to a previous recombinant gene;

(5) repeating (3) and (4), as necessary, until the further recombinant gene confers a desired level of optimized ability to express the gene product.

8.4.4. METHOD TO EVOLVE A BIOSENSOR

A further aspect of the invention is a method of evolving a biosensor for a compound A of interest, the method comprising:

(1) recombining at least first and second DNA segments from at least one gene conferring ability to detect a related compound B, the segments differing from each other in at least two nucleotides, to produce a library of recombinant genes;

(2) screening at least one recombinant gene from the library that confers optimized ability to detect compound A relative to a wild type form of the gene;

(3) recombining at least a segment from the at least one recombinant gene with a further DNA segment from the at least one gene, the same or different from the first and second segments, to produce a further library of recombinant genes;

(4) screening at least one further recombinant gene from the further library of recombinant genes that confers optimized ability to detect compound A relative to a previous recombinant gene;

(5) repeating (3) and (4), as necessary, until the further recombinant gene confers a desired level of optimized ability to detect compound A.

8.5. FERMENTATION OF MICRO-ORGANISMS

The fermentation of microorganisms for the production of natural products is the oldest and most sophisticated application of biocatalysis. Industrial microorganisms effect the multistep conversion of renewable feedstocks to high value chemical products in a single reactor and in so doing catalyze a multi-billion dollar industry. Fermentation products range from fine and commodity chemicals such as ethanol, lactic acid, amino acids and vitamins, to high value small molecule pharmaceuticals, protein pharmaceuticals, and industrial enzymes. (See, e.g., McCoy (1998) C&EN 13-19) for an introduction to biocatalysis. Success in bringing these products to market and success in competing in the market depends on continuous improvement of the whole cell biocatalysts. Improvements include increased yield of desired products, removal of unwanted co-metabolites, improved utilization of inexpensive carbon and nitrogen sources, and adaptation to fermenter conditions, increased production of a primary metabolite, increased production of a secondary metabolite, increased tolerance to acidic

non-stochastic mutagenesis provides a means of accumulating multiple useful mutation per cycle and thus eliminate the inherent limitation of current strain improvement programs (SIPs).

DNA stochastic &/or non-stochastic mutagenesis provides recursive mutagenesis, recombination, and selection of DNA sequences. A key difference between DNA stochastic &/or non-stochastic mutagenesis-mediated recombination and natural sexual recombination is that DNA stochastic &/or non-stochastic mutagenesis effects both the pairwise (two parents) and the poolwise (multiple parents) recombination of parent molecules, as described Supra. Natural recombination is more conservative and is limited to pairwise recombination. In nature, pairwise recombination provides stability within a population by preventing large leaps in sequences or genomic structure that can result from poolwise recombination. However, for the purposes of directed evolution, poolwise recombination is appealing since the beneficial mutations of multiple parents can be combined during a single cross to produce a superior offspring. Poolwise recombination is analogous to the crossbreeding of inbred strains in classic strain improvement, except that the crosses occur between many strains at once. In essence, poolwise recombination is a sequence of events that effects the recombination of a population of nucleic acid sequences that results in the generation of new nucleic acids that contains genetic information from more than two of the original nucleic acids. The power of in vitro DNA stochastic &/or non-stochastic mutagenesis is that large combinatorial libraries can be generated from a small pool of DNA fragments stochastic &/or non-stochastic mutagenized by recursive pairwise annealing and extension reactions, "matings." Many of the in vivo recombination formats described (such as plasmid-plasmid, plasmid-chromosome, phage-phage, phage-chromosome, phage-plasmid, conjugal DNA-chromosome, exogenous DNA-chromosome, chromosome-chromosome, with the DNA being introduced into the cell by natural and non-natural competence, transduction, transfection, conjugation, protoplast fusion, etc.) result primarily in the pairwise recombination of two DNA molecules. Thus, these formats when executed for only a single cycle of recombination are inherently limited in their potential to generate molecular diversity. To generate the level of diversity obtained by in vitro DNA stochastic

size, and each can be controlled and optimized. The uniform process of the Q-bot decreases human handling error and increases the rate of establishing cultures (roughly 10,000/4 hours). These cultures are then shaken in a temperature and humidity controlled incubator. The glass balls act to promote uniform aeration of cells and the dispersal of mycelial fragments similar to the blades of a fermenter.

1. Prescreen The ability to detect a subtle increase in the performance of a mutant over that of a parent strain relies on the sensitivity of the assay. The chance of finding the organisms having an improvement is increased by the number of individual mutants that can be screened by the assay. To increase the chances of identifying a pool of sufficient size a prescreen that increases the number of mutants processed by 10-fold can be used. The goal of the primary screen will be to quickly identify mutants having equal or better product titres than the parent strain(s) and to move only these mutants forward to liquid cell culture. The primary screen is an agar plate screen is analyzed by the Q-bot colony picker. Although assays can be fundamentally different, many result, e.g. , in the production of colony halos. For example, antibiotic production is assayed on plates using an overlay of a sensitive indicator strain, such as *B. subtilis*. Antibiotic production is typically assayed as a zone of clearing (inhibited growth of the indicator organism) around the producing organism. Similarly, enzyme production can be assayed on plates containing the enzyme substrate, with activity being detected as a zone of substrate modification around the producing colony. Product titre is correlated with the ratio of halo area to colony area.

The Q-bot or other automated system is instructed to only pick colonies having a halo ratio in the top 10% of the population i.e. 10,000 mutants from the 100,000 entering the plate prescreen. This increases the number of improved clones in the secondary assay and eliminates the wasted effort of screening knock-out and low producers. This improves the "hit rate" of the secondary assay.

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8.6.1.1 GENERAL TECHNIQUES

Thus, a general method for recursive sequence recombination for the embodiments herein is to begin with a gene encoding an enzyme or enzyme subunit and to evolve that gene either for ability to act on a new substrate, or for enhanced catalytic properties with an old substrate, either alone or in combination with other genes in a multistep pathway. The term "gene" is used herein broadly to refer to any segment or sequence of DNA associated with a biological function. Genes can be obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information, and may include sequences designed to have desired parameters. The ability to use a new substrate can be assayed in some instances by the ability to grow on a substrate as a nutrient source. In other circumstances such ability can be assayed by decreased toxicity of a substrate for a host cell, hence allowing the host to grow in the presence of that substrate. Biosynthesis of new compounds, such as antibiotics, can be assayed similarly by growth of an indicator organism in the presence of the host expressing the evolved genes. For example, when an indicator organism used in an overlay of the host expressing the evolved gene(s), wherein the indicator organism is sensitive or expected to be sensitive to the desired antibiotic, growth of the indicator organism would be inhibited in a zone around the host cell or colony expressing the evolved gene(s).

Another method of identifying new compounds is the use of standard analytical techniques such as mass spectroscopy, nuclear magnetic resonance, high performance liquid chromatography, etc. Recombinant microorganisms can be pooled and extracts or media supernatants assayed from these pools. Any positive pool can then be subdivided and the procedure repeated until the single positive is identified ("sib-selection").

In some instances, the starting material for recursive sequence recombination is a discrete gene, cluster of genes, or family of genes known or thought to be associated with



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8.6.1.1.5 CELL-CELL INDICTAOR

Only one component of the system, the library cells, is allowed to evolve. The screening is generally carried out in a two-dimensional immobilized format, such as on plates. The products of the metabolic pathways encoded by these genes (in this case, usually secondary metabolites such as antibiotics, polyketides, carotenoids, etc.) diffuse out of the library cell to the reporter cell. The product of the library cell may affect the reporter cell in one of a number of ways.

The assay system (indicator cell) can have a simple readout (e.g., green fluorescent protein, luciferase, β -galactosidase) which is induced by the library cell product but which does not affect the library cell. In these examples the desired product can be detected by colorimetric changes in the reporter cells adjacent to the library cell.

In other embodiments, indicator cells can in turn produce something that modifies the growth rate of the library cells via a feedback mechanism. Growth rate feedback can detect and accumulate very small differences. For example, if the library and reporter cells

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example, Evnin et al. (Proc. Natl. Acad. Sci. U.S.A. 87:6659-6663 (1990)) selected trypsin variants with altered substrate specificity by requiring that variant trypsin generate an essential amino acid for an arginine auxotroph by cleaving arginine -naphthylamide. This is thus a selection for arginine-specific trypsin, with the growth rate of the host being proportional to that of the enzyme activity.

The pool of cells surviving screening and/or selection is enriched for recombinant genes conferring the desired phenotype (e.g. altered substrate specificity, altered biosynthetic ability, etc.). Further enrichment can be obtained, if desired, by performing a second round of screening and/or selection without generating additional diversity.

The recombinant gene or pool of such genes surviving one round of screening/selection forms one or more of the substrates for a second round of recombination. Again, recombination can be performed in vivo or in vitro by any of the recursive sequence recombination formats described above.

If recursive sequence recombination is performed in vitro, the recombinant gene or genes to form the substrate for recombination should be extracted from the cells in which screening/selection was performed. Optionally, a subsequence of such gene or genes can be excised for more targeted subsequent recombination. If the recombinant gene(s) are contained within episomes, their isolation presents no difficulties. If the recombinant genes are chromosomally integrated, they can be isolated by amplification primed from known sequences flanking the regions in which recombination has occurred. Alternatively, whole genomic DNA can be isolated, optionally amplified, and used as the substrate for recombination. Small samples of genomic DNA can be amplified by whole genome amplification with degenerate primers (Barrett et al. *Nucleic Acids Research* 23:3488-3492 (1995)). These primers result in a large amount of random 3' ends, which can undergo homologous recombination when reintroduced into cells.

If the second round of recombination is to be performed *in vivo*, as is often the case, it can be performed in the cell surviving screening/selection, or the recombinant genes can be transferred to another cell type (e.g., a cell is type having a high frequency of

8.6.1.2 GENERAL METHODS

8.6.1.2.1 IN VITRO

In Vitro Formats one format for recursive sequence recombination in vitro is illustrated herein. The initial substrates for recombination are a pool of related sequences. The X's show where the sequences diverge. The sequences can be DNA or RNA and can be of various lengths depending on the size of the gene or DNA fragment to be recombined or stochastic &/or non-stochastic mutagenized. Preferably the sequences are from 50 bp to 100 kb.

The pool of related substrates are converted into overlapping fragments, e.g., from about 5 bp to 5 kb or more, as shown herein. Often, the size of the fragments is from about 10 bp to 1000 bp, and sometimes the size of the DNA fragments is from about 100 bp to 500 bp. The conversion can be effected by a number of different methods, such as DNaseI or RNase digestion, random shearing or partial restriction enzyme digestion.

Alternatively, the conversion of substrates to fragments can be effected by incomplete PCR amplification of substrates or PCR primed from a single primer. Alternatively, appropriate single-stranded fragments can be generated on a nucleic acid synthesizer. The concentration of nucleic acid fragments of a particular length and sequence is often less than 0.1 % or 1% by weight of the total nucleic acid. The number of different specific nucleic acid fragments in the mixture is usually at least about 100, 500 or 1000.

The mixed population of nucleic acid fragments are converted to at least partially single-stranded form. Conversion can be effected by heating to about 80C to 100C, more preferably from 90C to 96 C, to form single-stranded nucleic acid fragments and then reannealing. Conversion can also be effected by treatment with single-stranded DNA binding protein or recA protein. Single-stranded nucleic acid fragments having regions of sequence identity with other single-stranded nucleic acid fragments can then be reannealed by cooling to 4C to 75C, and preferably from 40C to 65C. Renaturation can be accelerated



by the addition of polyethylene glycol (PEG), other volume-excluding reagents or salt. The salt concentration is preferably from 0 mM to 200 mM more preferably the salt concentration is from 10 mM to 100 mM. The salt may be KCl or NaCl. The concentration of PEG is preferably from 0% to 20%, more preferably from 5% to 10%. The fragments that reanneal can be from different substrates as shown herein. The annealed nucleic acid fragments are incubated in the presence of a nucleic acid polymerase, such as Taq or Klenow, or proofreading polymerases, such as pfu or pwo, and dNTP's (i.e. dATP, dCTP, dGTP and dTTP). If regions of sequence identity are large, Taq polymerase can be used with an annealing temperature of between 45-65C. If the areas of identity are small, Klenow polymerase can be used with an annealing temperature of between 20-30T (Stemmer, Proc. Natl. Acad. Sci. USA (1994), supra). The polymerase can be added to the random nucleic acid fragments prior to annealing, simultaneously with annealing or after annealing.

The process of denaturation, renaturation and incubation in the presence of polymerase of overlapping fragments to generate a collection of polynucleotides containing different permutations of fragments is sometimes referred to as stochastic &/or non-stochastic mutagenesis of the nucleic acid in vitro. This cycle is repeated for a desired number of times. Preferably the cycle is repeated from 2 to 100 times, more preferably the sequence is repeated from 10 to 40 times. The resulting nucleic acids are a family of double-stranded polynucleotides of from about 50 bp to about 100 kb, preferably from 500 bp to 50 kb, as shown herein. The population represents variants of the starting substrates showing substantial sequence identity thereto but also diverging at several positions. The population has many more members than the starting substrates. The population of fragments resulting from stochastic &/or non-stochastic mutagenesis is used to transform host cells, optionally after cloning into a vector.

8.6.1.2.1.1 FULL LENGTH SEQUENCES

In a variation of in vitro stochastic &/or non-stochastic mutagenesis, subsequences of recombination substrates can be generated by amplifying the full-length sequences



under conditions which produce a substantial fraction, typically at least 20 percent or more, of incompletely extended amplification products. The amplification products, including the incompletely extended amplification products are denatured and subjected to at least one additional cycle of reannealing and amplification. This variation, wherein at least one cycle of reannealing and amplification provides a substantial fraction of incompletely extended products, is termed "stuttering." In the subsequent amplification round, the incompletely extended products anneal to and prime extension on different sequence-related template species.

8.6.1.2.1.2 OVERLAPPING SINGLE STRANDED DNA FRAGMENTS

In a further variation, at least one cycle of amplification can be conducted using a collection of overlapping single-stranded DNA fragments of related sequence, and different lengths. Each fragment can hybridize to and prime polynucleotide chain extension of a second fragment from the collection, thus forming sequence-recombined polynucleotides. In a further variation, single-stranded DNA fragments of variable length can be generated from a single primer by Vent DNA polymerase on a first DNA template. The single stranded DNA fragments are used as primers for a second, Kunkel-type template, consisting of a uracil-containing circular single-stranded DNA. This results in multiple substitutions of the first template into the second (see Levichkin et al. Mol. Biology 29:572-577 (1995)).

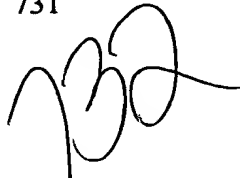
8.6.1.2.1.3 GENE CLUSTERS

Gene clusters such as those involved in polyketide synthesis (or indeed any multi-enzyme pathways catalyzing is analogous metabolic reactions) can be recombined by recursive sequence recombination even if they lack DNA sequence homology. Homology can be introduced using synthetic oligonucleotides as PCR primers. In addition to the specific sequences for the gene being amplified, all of the primers used to amplify one type of enzyme (for example the acyl carrier protein in polyketide synthesis) are

synthesized to contain an additional sequence of 20-40 bases 5' to the gene (sequence A) and a different 20-40 base sequence 3' to the gene (sequence B). The adjacent gene (in this case the keto- synthase) is amplified using a 5' primer which contains the complementary strand of sequence B (sequence B'), and a 3' primer containing a different 20-40 base sequence (C). Similarly, primers for the next adjacent gene (keto- reductases) contain sequences C' (complementary to C) and D. If 5 different polyketide gene clusters are being stochastic &/or non-stochastic mutagenized, all five acyl carrier proteins are flanked by sequences A and B following their PCR amplification. In this way, small regions of homology are introduced, making the gene clusters into site specific recombination cassettes. Subsequent to the initial amplification of individual genes, the amplified genes can then be mixed and subjected to primerless PCR. Sequence B at the 3' end of all of the five acyl carrier protein genes can anneal with and prime DNA synthesis from sequence B' at the 5' end of all five keto reductase genes. In this way all possible combinations of genes within the cluster can be obtained. Oligonucleotides allow such recombinants to be obtained in the absence of sufficient sequence homology for recursive sequence recombination described above. Only homology of function is required to produce functional gene clusters.

8.6.1.2.1.4 MULTI SUBUNIT ENZYMES

This method is also useful for exploring permutations of any other multi-subunit enzymes. An example of such enzymes composed of multiple polypeptides that have shown novel functions when the subunits are combined in novel ways are dioxygenases. Directed recombination between the four protein subunits of biphenyl and toluene dioxygenases produced functional dioxygenases with increased activity against trichloroethylene (Furukawa et. al. J. Bacteriol. 176: 2121-2123 (1994)). This combination of subunits from the two dioxygenases could also have been produced by cassette-stochastic &/or non-stochastic mutagenesis of the dioxygenases as described above, followed by selection for degradation of trichloroethylene.



In some polyketide synthases, the separate functions of the acyl carrier protein, keto-synthase, keto- reductase, etc. reside in a single polypeptide. In these cases domains within the single polypeptide may be stochastic &/or non-stochastic mutagenized, even if sufficient homology does not exist naturally, by introducing regions of homology as described above for entire genes. In this case, it may not be possible to introduce additional flanking sequences to the domains, due to the constraint of maintaining a continuous open reading frame.

Instead, groups of oligonucleotides are synthesized that are homologous to the 3' end of the first domain encoded by one of the genes to be stochastic &/or non-stochastic mutagenized, and the 5' ends of the second domains encoded by all of the other genes to be stochastic &/or non-stochastic mutagenized together. This is repeated with all domains, thus providing sequences that allow recombination between protein domains while maintaining their order.

8.6.1.2.1.5 CASSETTE-BASED

The cassette-based recombination method can be combined with recursive sequence recombination by including gene fragments (generated by DNase, physical shearing, DNA stuttering, etc.) for one or more of the genes. Thus, in addition to different combinations of entire genes within a cluster (e.g., for polyketide synthesis), individual genes can be stochastic &/or non-stochastic mutagenized at the same time (e.g., all acyl carrier protein genes can also be provided as fragmented DNA), allowing a more thorough search of sequence space.

8.6.1.2.1.6 IN VITRO WHOLE GENOME STOCHASTIC &/OR NON-STOCHASTIC MUTAGENESIS

The stochastic &/or non-stochastic mutagenesis of large DNA sequences, such as eukaryotic chromosomes, is difficult by prior art in vitro stochastic &/or non-stochastic mutagenesis methods. A method for overcoming this limitation is described herein.

will contain a combinatorial mixture of all possible ligations of fragments having homologous overhanging termini. A subset of this population will be complete chimeric chromosomes.

To screen the stochastic &/or non-stochastic mutagenized library, the chromosomes are delivered to a suitable host in a manner allowing for the uptake and expression of entire chromosomes. For example, YACs (yeast artificial chromosomes) can be delivered to eukaryotic cells by protoplast fusion.

Thus, the reassemble library could be encapsulated in liposomes and fused with protoplasts of the appropriate host cell. The resulting transformants would be propagated and screened for the desired cellular improvements. Once an improved population was identified, the chromosomes would be isolated, stochastic &/or non-stochastic mutagenized, and screened recursively.

8.6.1.2.1.7 WHOLE GENOME STOCHASTIC &/OR NON-STOCHASTIC MUTAGENESIS OF NATURALLY COMPETENT MICROORGANISMS

Natural competence is a phenomenon observed for some microbial species whereby individual cells take up DNA from the environment and incorporate it into their genome by homologous recombination. *Bacillus subtilis* and *Acetinetobacter Spp.* are known to be particularly efficient at this process. A method for the whole genome stochastic &/or non-stochastic mutagenesis of these and analogous organisms is described employing this process.

One goal of whole genome stochastic &/or non-stochastic mutagenesis is the rapid accumulation of useful mutations from a population of individual strains into one superior strain. If the organisms to be evolved are naturally competent, then a split pooled strategy for the recursive transformation of naturally competent cells with DNA originating from the pool will effect this process. An example procedure is as follows.

A population of naturally competent organisms that demonstrates a variety of useful traits (such as increased protein secretion) is identified. The strains are pooled, and the pool is split. One half of the pool is used as a source of gDNA, while the other is used to generate a pool of naturally competent cells.

The competent cells are grown in the presence of the pooled gDNA to allow DNA uptake and recombination. Cells of one genotype uptake and incorporate gDNA from cells of a different type generating cells having chimeric genomes. The result is a population of cells representing a combinatorial mixture of the genetic variations originating in the original pool. These cells are pooled again and transformed with the same source of DNA again. This process is carried out recursively to increase the diversity of the genomes of cells resulting from transformation. Once sufficient diversity has been generated, the cell population is screened for new chimeric organisms demonstrating desired improvements.

This process is enhanced by increasing the natural competence of the host organism. COMS is a protein that, when expressed in *B. subtilis*, enhances the efficiency of natural competence mediated transformation more than an order of magnitude.

It was demonstrated that approximately 100% of the cells harboring the plasmid pCOMS uptake and recombine genomic DNA fragments into their genomes. In general, approximately 10% of the genome is recombined into any given transformed cell. This observation was demonstrated by the following.

A strain of *B. subtilis* pCOMS auxotrophic for two nutritional markers was transformed with genomic DNA (gDNA) isolated from a prototrophic strain of the same organism. 10% of the cells exposed to the DNA were prototrophic for one of the two nutrient markers. The average size of the DNA strand taken up by *B. subtilis* is approximately 50kb or about 2% of the genome. Thus 1 of every ten cells had recombined a marker that was represented 1 in every fifty molecules of uptaken gDNA. Thus, most of the cells take up and recombine with approximately five 50kb molecules or 10% of the

genome. This method represents a powerful tool for rapidly and efficiently recombining whole microbial genomes.

In the absence of pCOMS, only 0.3% of the cells prepared for natural competency uptake and integrate a specific marker. This suggested that about 15% of the cells actually underwent recombination with a single genomic fragment. Thus, a recursive transformation strategy as described above produces a whole genome stochastic &/or non-stochastic mutagenized library, even in the absence of pCOMS. In the absence of pCOMS, however, the complex genomes will represent a smaller, but still screenable percentage of the transformed or stochastic &/or non-stochastic mutagenized population.

8.6.1.2.1.8 CONGRESSION

Congression is the integration of two independent unlinked markers into a cell. 0.3% of naturally competent *B. subtilis* cells integrate a single marker (described above). Of these, about 10% have taken up an additional marker. Thus, if one selects or screens for the integration of one specific marker, 10% of the resulting population will have integrated another specific marker. This provides a way of enriching for specific integration events.

For example, if one is looking for the integration of a gene for which there is no easy screen or selection, it will exist as 0.3% of the cell population. If the population is first selected for a specific integration event, then the desired integration will be found in 10% of the population. This represents a significant (about 30-fold) enrichment for the desired event. This enrichment is defined as the "congression effect." The congression effect is not influenced by the presence of pCOMS, thus the "pCOMS effect" is simply to increase the percentage of naturally competent cells that are truly naturally competent from about 15% in its absence to 100% in its presence. All competent cells still uptake about the same amount of DNA or about 10% of the *Bacillus* genome.

The congression effect can be used in the following examples to enhance whole genome stochastic &/or non-stochastic mutagenesis as well, as the targeted integration of stochastic &/or non-stochastic mutagenized genes to the chromosome.

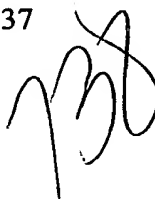
8.6.1.2.1.9 B.SUBTILIS STOCHASTIC &/OR NON-STOCHASTIC MUTAGENESIS

A population of B. subtilis cells having desired properties are identified, pooled and stochastic &/or non-stochastic mutagenized as described above with one exception: once the pooled population is split, half of the population is transformed with an antibiotic selection marker that is flanked by sequence that targets its integration and disruption of a specific nutritional gene, for example, one involved in amino biosynthesis. Transformants resistant to the drug are auxotrophic for that nutrient. The resistant population is pooled and grown under conditions rendering them naturally competent (or optionally first transformed with pCOMS).

The competent cells are then transformed with gDNA isolated from the original pool, and prototrophs are selected. The prototrophic population will have undergone recombination with genomic fragments encoding a functional copy of the nutritional marker, and thus will be enriched for cells having undergone recombination at other genetic loci by the congression effect.

8.6.1.2.1.10 TARGETING OF GENES AND GENE LIBRARIES TO THE CHROMOSOME

It is useful to be able to efficiently deliver genes or gene libraries directly to a specific location in a cells chromosome. As above, target cells are transformed with a positive selection marker flanked by sequences that target its homologous recombination into the chromosome. Selected cells harboring the marker are made naturally competent (with or without pCOMS, but preferably the former) and transformed with a mixture of



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Haas in Bacterial Conjugation (Clewett, ed., Plenum Press, New York 1993), at pp. 137-188 (incorporated by reference in their entirety for all purposes). Conjugation occurs between many types of gram negative bacteria, and some types of gram positive bacteria. Conjugative transfer is also known between bacteria and plant cells (*Agrobacterium tumefaciens*) or yeast. As discussed in patent 5,837,458, the genes responsible for conjugative transfer can themselves be evolved to expand the range of cell types (e.g., from bacteria to mammals) between which such transfer can occur.

Conjugative transfer is effected by an origin of transfer (*oriT*) and flanking genes (MOB A, B and C, and 15-25 genes, termed *tra*, encoding the structures and enzymes necessary for conjugation to occur. The transfer origin is defined as the site required in *cis* for DNA transfer. *Tra* genes include *tra A, B, C, D, E, F, G, H, I, J, K, L, M, N, P, Q, R, S, T, U, V, W, X, Y, Z, virAB* (alleles I-II), *C, D, E, G, IHF*, and *FinOP*. *Tra* genes can be expressed in *cis* or *trans* to *oriT*. Other cellular enzymes, including those of the RecBCD pathway, RecA, SSB protein, DNA gyrase, DNA polII, and DNA ligase, are also involved in conjugative transfer. RecE or recF pathways can substitute for RecBCD.

One structural protein encoded by a *tra* gene is the sex pilus, a filament constructed of an aggregate of a single polypeptide protruding from the cell surface. The sex pilus binds to a polysaccharide on recipient cells and forms a conjugative bridge through which DNA can transfer. This process activates a site-specific nuclease encoded by a MOB gene, which specifically cleaves DNA to be transferred at *oriT*. The cleaved DNA is then threaded through the conjugation bridge by the action of other *tra* enzymes.

Mobilizable vectors can exist in episomal form or integrated into the chromosome. Episomal mobilizable vectors can be used to exchange fragments inserted into the vectors between cells. Integrated mobilizable vectors can be used to mobilize adjacent genes from the chromosome.

8.6.1.2.1.12 USE OF INTEGRATED MOBILIZED VECTORS TO PROMOTE EXCHANGE OF GENOMIC DNA

The F plasmid of *E. coli* integrates into the chromosome at high frequency and mobilizes genes unidirectional from the site of integration (Clewell, 1993, supra; Firth et al., in *Escherichia coli* and *Salmonella* Cellular and Molecular Biology 2, 23 77-2401 (1996); Frost et al., *Microbiol. Rev.* 58, 162-210 (1994)). Other mobilizable vectors do not spontaneously integrate into a host chromosome at high efficiency, but can be induced to do so by growth under particular conditions (e.g., treatment with a mutagenic agent, growth at a nonpermissive temperature for plasmid replication). See Reimann & Haas in *Bacterial Conjugation* (ed. Clewell, Plenum Press, NY 1993), Ch. 6. Of particular interest is the IncP group of conjugal plasmids which are typified by their broad host range (Clewell, 1993, supra. Donor "male" bacteria which bear a chromosomal insertion of a conjugal plasmid, such as the *E. coli* F factor can efficiently donate chromosomal DNA to recipient "female" enteric bacteria which lack F (F-). Conjugal transfer from donor to recipient is initiated at *oriT*. Transfer of the nicked single strand to the recipient occurs in a 5' to 3' direction by a rolling circle mechanisms which allows mobilization of tandem chromosomal copies. Upon entering the recipient, the donor strand is discontinuously replicated. The linear, single-stranded donor DNA strand is a potent substrate for initiation of *recA*-mediated homologous recombination within the recipient. Recombination between the donor strand and recipient chromosomes can result in the inheritance of donor traits. Accordingly, strains which bear a chromosomal copy of F are designated Hfr (for high frequency of recombination) (Low, 1996 in *Escherichia coli* and *Salmonella* Cellular and Molecular Biology Vol. 2, pp. 2402- 2405; Sanderson, in *Escherichia coli* and *Salmonella* Cellular and Molecular Biology 2, 2406-2412 (1996)).

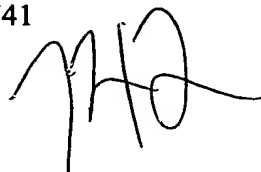
The ability of strains with integrated mobilizable vector to transfer chromosomal DNA provides a rapid and efficient means of exchanging genetic material between a population of bacteria thereby allowing combination of positive mutations and dilution of negative mutations. Such stochastic &/or non-stochastic mutagenesis methods typically start with a population of strains with an integrated mobilizable vector encompassing at least some genetic diversity.

The genetic diversity can be the result of natural variation, exposure to a mutagenic agent or introduction of a fragment library. The population of cells is cultured without selection to allow genetic exchange, recombination and expression of recombinant genes. The cells are then screened or selected for evolution toward a desired property. The population surviving selection/screening can then be subject to a further round of stochastic &/or non-stochastic mutagenesis by IVR-mediated genetic exchange, or otherwise.

The natural efficiency of Hfr and other strains with integrated mob vectors as recipients of conjugal transfer can be improved by several means. The relatively low recipient efficiency of natural BFR strains is attributable to the products of *traS* and *traT* genes of F (Clewett, 1993, supra; Firth et al., 1996, supra.- Frost et al., 1994, supra; Achtman et al., J Mol. Biol. 138, 779-795 (1980). These products are localized to the inner and outer membranes of F+ strains, respectively, where they serve to inhibit redundant matings between two strains which are both capable of donating DNA. The effects of *traS* and *traT*, and cognate genes in other conjugal plasmids, can be eliminated by use of knockout cells incapable of expressing these enzymes or reduced by propagating cells on a carbon- limited source. (Peters et al., J Bacteriol., 178, 3037-3043 (1996)).

In some methods, the starting population of cells has a mobilizable vector integrated at different genomic sites. Directional transfer from *oriT* typically results in more frequent inheritance of traits proximal to *oriT*. This is because mating pairs are fragile and tend to dissociate (particularly when in liquid medium) resulting in the interruption of transfer.

In a population of cells having a mobilizable vector integrated at different sites, chromosomal exchange occurs in a more random fashion. Kits of Hfr strains are available from the E coli. Genetic Stock Center and the Salmonella Genetic Stock Centre (Frost et al., 1994, supra).



Alternatively, a library of strains with oriT at random sites and orientations can be produced by insertion mutagenesis using a transposon which bears oriT. The use of a transposon bearing an oriT [e.g., the Tn5-oriT described by Yakobson EA, et al. J. Bacteriol. 1984 Oct; 160(1): 451-453] provides a quick method of generating such a library. Transfer functions for mobilization from the transposon-borne oriT sites are provided by a helper vector in trans. It is possible to generate similar genetic constructs using other sequences known to one of skill as well.

In one aspect, a recursive scheme for genomic stochastic &/or non-stochastic mutagenesis using Tn-oriT elements is provided. A prototrophic bacterial strain or set of related strains bearing a conjugal plasmid, such as the F fertility factor or a member of the IncP group of broad host range plasmids is mutagenized and screened for the desired properties. Individuals with the desired properties are mutagenized with a Tn-oriT element and screened for acquisition of an auxotrophy (e.g., by replica-plating to a minimal and complete media) resulting from insertion of the Tn-oriT element in any one of many biosynthetic gene scattered across the genome. The resulting auxotrophs are pooled and allowed to mate under conditions promoting male-to-male matings, e.g., during growth in close proximity on a filter membrane. Note that transfer functions are provided by the helper conjugal plasmid present in the original strain set. Recombinant transconjugants are selected on minimal medium and screened for further improvement.

Optionally, strains bearing integrated mobilizable vectors are defective in mismatch repair gene(s). Inheritance of donor traits which arise from sequence heterologies increases in strains lacking the methyl-directed mismatch repair system. Optionally, the gene products which decrease recombination efficiency can be inhibited by small molecules.

Intergenic conjugal transfer between species such as *E. coli* and *Salmonella typhimurium*, which are 20% divergent at the DNA level, is also possible if the recipient strain is mutH, mutL or mutS (see Rayssiguier et al., Nature 342, 396-401 (1989)). Such

transfer can be used to obtain recombination at several points as shown by the following example.

One example uses an *S. typhimurium* Hfr donor strain having markers thr557 at map position 0, pyrF2690 at 33 min, serA13 at 62 min and hfrK5 at 43 min. MutS +/-, F- *E. coli* recipient strains had markers pyrD68 at 21 min aroC355 at 51 min, ilv3164 at 85 min and mutS215 at 59 min. The triauxotrophic *S. typhimurium* Hfr donor and isogenic mutS +/- triauxotrophic *E. coli* recipient were inoculated into 3 ml of Lb broth and shaken at 37C until fully grown. 100 ul of the donor and each recipient were mixed in 10 ml fresh LB broth, and then deposited to a sterile Millipore 0.45 uM HA filter using a Nalgene 250 ml reusable filtration device. The donor and recipients alone were similarly diluted and deposited to check for reversion. The filters with cells were placed cell-side-up on the surface of an LB agar plate which was incubated overnight at 37C. The filters were removed with the aid of a sterile forceps and placed in a sterile 50 ml tube containing 5 ml of minimal salts broth. Vigorous vortexing was used to wash the cells from the filters. 100 ul of mating mixtures, as well as donor and recipient controls were spread to LB for viable cell counts and minimal glucose supplemented with either two of the three recipient requirements for single recombinant counts, one of the three requirements for double recombinant counts, or none of the three requirements for triple recombinant counts. The plates were incubated for 48 hr at 37C after which colonies were counted.

Frequencies are further enhanced by increasing the ratio of donor to recipient cells, or by repeatedly mating the original donor strains with the previously generated recombinant progeny.

8.6.1.2.1.13 INTRODUCTION OF FRAGMENTS BY CONJUGATION

Sobilizable vectors can also be used to transfer fragment libraries into cells to be evolved. This approach is particularly useful in situations in which the cells to be evolved cannot be efficiently transformed directly with the fragment library but can undergo conjugation with primary cells that can be transformed with the fragment library. DNA

be combined with other methods of stochastic &/or non-stochastic mutagenesis.

8.6.1.2.1.14 GENETIC EXCHANGE PROMOTED BY TRANSDUCING PHAGE IN CELLS SUSEPTIBLE TO PHAGE

Phage transduction can include the transfer, from one cell to another, of nonviral genetic material within a viral coat (Masters, in *Escherichia coli* and *Salmonella Cellular and Molecular Biology* 2, 2421-2442 (1996). Perhaps the two best examples of generalized transducing phage are bacteriophages P I and P22 of *E. coli* and *S. typhimurium*, respectively. Generalized transducing bacteriophage particles are formed at a low frequency during lytic infection when viral-genome-sized, doubled-stranded fragments of host (which serves as donor) chromosomal DNA are packaged into phage heads. Promiscuous high transducing (HT) mutants of bacteriophage P22 which efficiently package DNA with little sequence specificity have been isolated. Infection of a susceptible host results in a lysate in which up to 50% of the phage are transducing particles. Adsorption of the generalized transducing particle to a susceptible recipient cell results in the injection of the donor chromosomal fragment. RecA-mediated homologous recombination following injection of the donor fragment can result in the inheritance of donor traits. Another type of phage which achieves quasi random insertion of DNA into the host chromosome is Mu. For an overview of Mu biology, see, Groisman (1991) in *Methods in Enzymology* v. 204. Mu can generate a variety of chromosomal rearrangements including deletions, inversions, duplications and transpositions. In addition, elements which combine the features of P22 and Mu are available, including Mud-P22, which contains the ends of the Mu genome in place of the P22 att site and int gene. See, Berg, *supra*.

Generalized transducing phage can be used to exchange genetic material between a population of cells encompassing genetic diversity and susceptible to infection by the phage. Genetic diversity can be the result of natural variation between cells, induced mutation of cells or the introduction of fragment libraries into cells. DNA is then exchanged between cells by generalized transduction. If the phage does not cause lysis of

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cells, the entire population of cells can be propagated in the presence of phage. If the phage results in lytic infection, transduction is performed on a split pool basis. That is, the starting population of cells is divided into two. One subpopulation is used to prepare transducing phage. The transducing phage are then infected into the other subpopulation. Preferably, infection is performed at high multiplicity of phage per cell so that few cells remain uninfected. Cells surviving infection are propagated and screened or selected for evolution toward a desired property. The pool of cells surviving screening/selection can then be stochastic &/or non-stochastic mutagenized by a further round of generalized transduction or by other stochastic &/or non-stochastic mutagenesis methods. Recursive split pool transduction is optionally performed prior to selection to increase the diversity of any population to be screened.

The efficiency of the above methods can be increased by reducing infection of cells by infectious (nontransducing phage) and by reducing lysogen formation. The former can be achieved by inclusion of chelators of divalent cations, such as citrate and EDTA in culture media. Tail defective transducing phages can be used to allow only a single round of infection.

Divalent cations are required for phage absorption and the inclusion of chelating agents therefore provides a means of preventing unwanted infection. Integration defective (int) derivatives of generalized transducing phage can be used to prevent lysogen formation. In a further variation, host cells with defects in mismatch repair gene(s) can be used to increase recombination between transduced DNA and genomic DNA.

8.6.1.2.1.15 USE OF LOCKED IN PROPHAGES TO FACILITATE DNA STOCHASTIC &/OR NON-STOCHASTIC MUTAGENESIS

The use of a hybrid, mobile genetic element (locked-in prophages) as a means to facilitate whole genome stochastic &/or non-stochastic mutagenesis of organisms using phage transduction as a means to transfer DNA from donor to recipient is a preferred

embodiment. One such element (Mud-P22) based on the temperate Salmonella phage P22 has been described for use in genetic and physical mapping of mutations. See, Youderian et al. (1988) Genetics 118:581 - 592, and Benson and Goldman (1992) J Bacteriol. 174(5):1673-1681. Individual Mud-P22 insertions package specific regions of the Salmonella chromosome into phage P22 particles.

Libraries of random Mud-P22 insertions can be readily isolated and induced to create pools of phage particles packaging random chromosomal DNA fragments. These phage particles can be used to infect new cells and transfer the DNA from the host into the recipient in the process of transduction. Alternatively, the packaged chromosomal DNA can be isolated and manipulated further by techniques such as DNA stochastic &/or non-stochastic mutagenesis or any other mutagenesis technique prior to being reintroduced into cells (especially recD cells for linear DNA) by transformation or electroporation, where they integrate into the chromosome. Either the intact transducing phage particles or isolated DNA can be subjected to a variety of mutagens prior to reintroduction into cells to enhance the mutation rate.

Mutator cell lines such as mutD can also be used for phage growth. Either method can be used recursively in a process to create genes or strains with desired properties. E. coli cells carrying a cosmid clone of Salmonella LPS genes are infectable by P22 phage. It is possible to develop similar genetic elements using other combinations of transposable elements and bacteriophages or viruses as well. P22 is a lambdoid phage that packages its DNA into stochastic &/or non-stochastic mutagenized phage particles (heads) by a "headful" mechanism. Packaging of phage DNA is initiated at a specific site (pac) and proceeds unidirectionally along a linear, double stranded normally concatameric molecule. When the phage head is full (about 43 kb), the DNA strand is cleaved, and packaging of the next phage head is initiated. Locked-in or excision-defective P22 prophages, however, initiate packaging at their pac site, and then proceed unidirectionally along the chromosome, packaging successive headfuls of chromosomal DNA (rather than phage DNA). When these transducing phages infect new Salmonella cells they inject the chromosomal DNA from the original host into the recipient cell, where it can recombine

Alternatively, the gene/operon of interest can be readily moved from a mutagenized/stochastic &/or non-stochastic mutagenized host into a different background to screen/select for modifications in the gene/operon itself. It is also possible to develop similar genetic elements using other combinations of transposable elements and bacteriophages or viruses as well. Similar systems are set up in other organisms, e.g., that do not allow replication of P22 or P1. Broad host range phages and transposable elements are especially useful. Similar genetic elements are derived from other temperate phages that also package by a headful mechanism. In general, these are the phages that are capable of generalized transduction. Viruses infecting eukaryotic cells may be adapted for similar purposes. Examples of generalized transducing phages that are useful are described in: Green et al., "Isolation and preliminary characterization of lytic and lysogenic phages with wide host range within the streptomycetes", J Gen Microbiol 131(9):2459-2465 (1985); Studdard et al., "Genome structure in Streptomyces spp.: adjacent genes on the S. coelicolor A3(2) linkage map have cotransducible analogs in S. venezuelae", J Bacteriol 169(8):3 814-3 816 (1987); Wang et al., "High frequency generalized transduction by miniMu plasmid phage", Genetics 116(2):201-206, (1987); Welker, N. E., "Transduction in Bacillus stearothermophilus", J Bacteriol, 176(11):3354-3359, (1988); Darzins et al., "Mini-D3112 bacteriophage transposable elements for genetic analysis of Pseudomonas aeruginosa, J Bacteriol 171(7):3909-3916 (1989); Hugouvieux-Cotte-Pattat et al., "Expanded linkage map of Erwinia chrysanthemi strain 3937", Mol Microbiol 3(5):573-581, (1989); Ichige et al. "Establishment of gene transfer systems for and construction of the genetic map of a marine Vibrio strain", J Bacteriol 171(4):1825-1834 (1989); Murainatsu et al., "Two generalized transducing phages in Vibrio parahaemolyticus and Vibrio alginolyticus", Microbiol Immunol (12):1073-1084 (1991); Regue et al., "A generalized transducing bacteriophage for Serratia marcescens", Res Microbiol 42(1):23 - 27, (199 1) - Kiesel et al , "Phage Acn I -mediated transduction in the facultatively methanol-utilizing A cetobacter methanolicus MB 58/4", J Gen Virol 74(9):1741-1745 (1993); Blahova et al., "Transduction of imipenem resistance by the phage F- 116 from a nosocomial strain of Pseudomonas aeruginosa isolated in Slovakia", Acta Virol 38(5):247-250 (1994); Kidambi et al., "Evidence for phage- mediated gene transfer among Pseudomonas aeruginosa strains on the phylloplane", Appl Environ Microbiol 60:(2)496-

non-homologous recombination, followed by selection or screening for the desired phenotype, is a preferred way of identifying optimal chromosomal positions for expression of a target. This strategy is illustrated herein.

A variety of transposon mediated delivery systems can be employed to deliver genes of interest, either individual genes, genomic libraries, or a library of stochastic &/or non-stochastic mutagenized gene(s) randomly throughout the genome of a host. Thus, in one preferred embodiment, the improvement of a cellular function is achieved by cloning a gene of interest, for example a gene encoding a desired metabolic pathway, within a transposon delivery vehicle.

Such transposon vehicles are available for both Gram-negative and Gram-positive bacteria. De Lorenzo and Timis (1994) *Methods in Enzymology* 235:385-404 describe the analysis and construction of stable phenotypes in gram-negative Bacteria with Tn5- and Tn 10-derived minitransposons. Kleckner et al. (1991) *Methods in Enzymology* 204, chapter 7 describe uses of transposons such as Tn 10, including for use in gram positive bacteria. Petit et al. (1990) *Journal of Bacteriology*, 172(12):6736-6740 describe Tn10 derived transposons active in *Bacillus Subtilis*. The transposon delivery vehicle is introduced into a cell population, which is then selected for recombinant cells that have incorporated the transposon into the genome.

The selection is typically by any of a variety of drug resistant markers also carried within the transposon. The selected subpopulation is screened for cells having improved expression of the gene(s) of interest. Once cells harboring the genes of interest in the optimal location are isolated, the genes are amplified from within the genome using PCR, stochastic &/or non-stochastic mutagenized, and cloned back into a similar transposon delivery vehicle which contains a different selection marker within the transposon and lacks the transposon integrase gene.

This stochastic &/or non-stochastic mutagenized library is then transformed back into the strain harboring the original transposon, and the cells are selected for the presence

of the new resistance marker and the loss of the previous selection marker. Selected cells are enriched for those that have exchanged by homologous recombination the original transposon for the new transposon carrying members of the stochastic &/or non-stochastic mutagenized library. The surviving cells are then screened for further improvements in the expression of the desired phenotype. The genes from the improved cells are then amplified by the PCR and stochastic &/or non-stochastic mutagenized again. This process is carried out recursively, oscillating each cycle between the different selection markers. Once the gene(s) of interest are optimized to a desired level, the fragment can be amplified and again randomly distributed throughout the genome as described above to identify the optimal location of the improved genes.

Alternatively, the gene(s) conferring a desired property may not be known. In this case the DNA fragments cloned within the transposon delivery vehicle could be a library of genomic fragments originating from a population of cells derived from one or more strains having the desired property(ies). The library is delivered to a population of cells derived from one or more strains having or lacking the desired property(ies) and cells incorporating the transposon are selected. The surviving cells are then screened for acquisition or improvement of the desired property. The fragments contained within the surviving cells are amplified by PCR and then cloned as a pool into a similar transposon delivery vector harboring a different selection marker from the first delivery vector. This library is then delivered to the pool of surviving cells, and the population having acquired the new selective marker is selected. The selected cells are then screened for further acquisition or improvement of the desired property.

In this way the different possible combinations of genes conferring or improving a desired phenotype are explored in a combinatorial fashion. This process is carried out repetitively with each new cycle employing an additional selection marker. Alternatively, PCR fragments are cloned into a pool of transposon vectors having different selective markers. These are delivered to cells and selected for 1, 2, 3, or more markers.

Alternatively, the amplified fragments from each improved cell are stochastic &/or

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this method, a genomic library is cloned into a suicide vector containing a selective marker that also at higher dosage provides an enhanced phenotype. An example of such a marker is the kanamycin resistance gene. At successively higher copy number, resistance to successively higher levels of kanamycin is achieved. The genomic library is delivered to a target cell by any of a variety of methods including transformation, transduction, conjugation, etc. Cells that have incorporated the vector into the chromosome by homologous recombination between the vector and chromosomal copies of the cloned genes can be selected by requiring expression of the selection marker under conditions where the vector does not replicate. This recombination event results in the duplication of the cloned DNA fragment in the host chromosome with a copy of the vector and selection marker separating the two copies. The population of surviving cells are screened for improvement of a desired cellular property resulting from the gene duplication event. Further gene duplication events resulting in additional copies of the original cloned DNA fragments can be generated by further propagating the cells under successively more stringent selective conditions i.e. increased concentrations of kanamycin. In this case selection requires increased copies of the selective marker, but increased copies of the desired gene fragment is also concomitant. Surviving cells are further screened for an improvement in the desired phenotype. The resulting population of cells likely resulted in the amplification of different genes since often many genes effect a given phenotype. To generate a library of the possible combinations of these genes, the original selected library showing phenotypic improvements are recombined, using the methods described herein, e.g., protoplast fusion, split pool transduction, transformation, conjugation, etc.

The recombined cells are selected for increased expression of the selective marker. Survivors are enriched for cells having incorporated additional copies of the vector sequence by homologous recombination, and these cells will be enriched for those having combined duplications of different genes. In other words, the duplication from one cell of enhanced phenotype becomes combined with the duplication of another cell of enhanced phenotype. These survivors are screened for further improvements in the desired phenotype. This procedure is repeated recursively until the desired level of phenotypic expression is achieved.

Alternatively, genes that have been identified or are suspected as being beneficial in increased copy number are cloned in tandem into appropriate plasmid vectors. These vectors are then transformed and propagated in an appropriate host organism. Plasmid-plasmid recombination between the cloned gene fragments result in further duplication of the genes. Resolution of the plasmid doublet can result in the uneven distribution of the gene copies, with some plasmids having additional gene copies and others having fewer gene copies. Cells carrying this distribution of plasmids are then screened for an improvement in the phenotype effected by the gene duplications.

In summary, a method of selecting for increased copy number of a nucleic acid sequence by the above procedure is provided. In the method, a genomic library in a suicide vector comprising a dose-sensitive selectable marker is provided, as noted above. The genomic library is transduced into a population of target cells. The target cells are selected in a population of target cells for increasing doses of the selectable marker under conditions in which the suicide vector does not replicate episomally. A plurality of target cells are selected for the desired phenotype, recombined and reselected. The process is recursively repeated, if desired, until the desired phenotype is obtained.

8.6.1.2.1.18 STRATEGIES FOR IMPROVING GENOMIC STOCHASTIC &/OR NON-STOCHASTIC MUTAGENESIS VIA TRANSFORMATION OF LINEAR DNA FRAGMENTS

Wild-type members of the Enterobacteriaceae (e.g., *Escherichia coli*) are typically resistant to genetic exchange following transformation of linear DNA molecules.

This is due, at least in part, to the Exonuclease V (Exo V) activity of the RecBCD holoenzyme which rapidly degrades linear DNA molecules following transformation. Production of ExoV has been traced to the *recD* gene, which encodes the D subunit of the holoenzyme. As demonstrated by Russel et al. (1989) *Journal of Bacteriology* 2609-2613,

homologous recombination between a transformed linear donor DNA molecule and the chromosome of recipient is readily detected in a strains bearing a loss of function mutation in a recD mutant.

The use of recD strains provides a simple means for genomic stochastic &/or non-stochastic mutagenesis of the Enterobacteriaceae. For example, a bacterial strain or set of related strains bearing a recD null mutation (e.g., the *E. coli* recD1903::mini-Tet allele) is mutagenized and screened for the desired properties. In a split-pool fashion, chromosomal DNA prepared on one aliquot could be used to transform (e.g., via electroporation or chemically induced competence) the second aliquot. The resulting transformants are then screened for improvement, or recursively transformed prior to screening.

The use of RecE/ recT as described supra, can improve homologous recombination of linear DNA fragments. The RecBCD holoezyme plays an important role in initiation of RecA-dependent homologous recombination. Upon recognizing a dsDNA end, the RecBCD enzyme unwinds and degrades the DNA asymmetrically in a 5' to 3' direction until it encounters a chi (or 'X')-site (consensus 5'-GCTGGTGG-3') which attenuates the nuclease activity. This results in the generation of a ssDNA terminating near the c site with a 3'-ssDNA tail that is preferred for RecA loading and subsequent invasion of dsDNA for homologous recombination. Accordingly, preprocessing of transforming fragments with a 5' to 3' specific ssDNA Exonuclease, such as Lamda () exonuclease (available, e.g., from Boeringer Mannheim) prior to transformation may serve to stimulate homologous recombination in recD- strain by providing ssDNA invasive end for RecA loading and subsequent strand invasion.

The addition of DNA sequence encoding chi-sites (consensus 5'-GCTGGTGG-3') to DNA fragments can serve to both attenuate Exonuclease V activity and stimulate homologous recombination, thereby obviating the need for a recD mutation (see also, Kowalczykowski, et al. (1994) "Biochemistry of homologous recombination in *Escherichia coli*," Microbiol. Rev. 58:401-465 and Jessen, et al. (1998) "Modification of bacterial artificial chromosomes through Chi-stimulated homologous recombination and

approach requires no sequence information, nor any knowledge or assumptions about the nature of protein or pathway interactions, or even of what steps are rate-limiting; it relies only on detection of the desired phenotype. This sort of random cloning and subsequent evolution by DNA stochastic &/or non-stochastic mutagenesis of positively interacting genomic sequences is extremely powerful and generic. A variety of sources of genomic DNA are used, from isogenic strains to more distantly related species with potentially desirable properties. In addition, the technique is applicable to any cell for which the molecular biology basics of transformation and cloning vectors are available, and for any property which can be assayed (preferably in a high-throughput format). Alternatively, once optimized, the evolved DNA can be returned to the chromosome by homologous recombination or randomly by phage mediated site-specific recombination.

8.6.1.2.1.20 HOMOLOGOUS RECOMBINATION WITHIN THE CHROMOSOME

Homologous recombination within the chromosome is used to circumvent the limitations of plasmid based evolution and size restrictions. The strategy is similar to that described above for stochastic &/or non-stochastic mutagenesis genes within their chromosomal context, except that no in vitro stochastic &/or non-stochastic mutagenesis occurs. Instead, the parent strain is treated with mutagens such as ultraviolet light or nitrosoguanidine, and improved mutants are selected. The improved mutants are pooled and split. Half of the pool is used to generate random genomic fragments for cloning into a homologous recombination vector. Additional genomic fragments are optionally derived from related species with desirable properties. The cloned genomic fragments are homologously recombined into the genomes of the remaining half of the mutant pool, and variants with improved properties are selected. These are subjected to a further round of mutagenesis, selection and recombination. Again this process is entirely generic for the improvement of any whole cell biocatalyst for which a recombination vector and an assay can be developed. Here again, it should be noted that recombination can be performed recursively prior to screening.

be described below, the plasmids include mobilization (MOB) functions. The substrates can be incorporated into the same or different plasmids. Often at least two different types of plasmid having different types of selectable markers are used to allow selection for cells containing at least two types of vector. Also, where different types of plasmid are employed, the different plasmids can come from two distinct incompatibility groups to allow stable co-existence of two different plasmids within the cell. Nevertheless, plasmids from the same incompatibility group can still co-exist within the same cell for sufficient time to allow homologous recombination to occur.

Plasmids containing diverse substrates are initially introduced into cells by any method (e.g., chemical transformation, natural competence, electroporation, biolistics, packaging into phage or viral systems). Often, the plasmids are present at or near saturating concentration (with respect to maximum transfection capacity) to increase the probability of more than one plasmid entering the same cell. The plasmids containing the various substrates can be transfected simultaneously or in multiple rounds. For example, in the latter approach cells can be transfected with a first aliquot of plasmid, transfectants selected and propagated, and then infected with a second aliquot of plasmid.

Having introduced the plasmids into cells, recombination between substrates to generate recombinant genes occurs within cells containing multiple different plasmids merely by propagating the cells. However, cells that receive only one plasmid are unable to participate in recombination and the potential contribution of substrates on such plasmids to evolution is not fully exploited (although these plasmids may contribute to some extent if they are propagated in mutator cells). The rate of evolution can be increased by allowing all substrates to participate in recombination. Such can be achieved by subjecting transfected cells to electroporation. The conditions for electroporation are the same as those conventionally used for introducing exogenous DNA into cells (e.g., 1,000-2,500 volts, 400 uF and a 1-2 mM gap). Under these conditions, plasmids are exchanged between cells allowing all substrates to participate in recombination.

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Table 1 The number of cases of COVID-19 by age group and sex in the United States, March 2020–June 2021

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Other viruses, such as the T-phage and lytic lambda, undergo recombination with the plasmid but ultimately kill the host S cell and destroy plasmid DNA. For viruses that infect cells without killing the host, cells containing recombinant plasmids and virus can be screened/selected using the same approach as for plasmid-plasmid recombination. Progeny virus extruded by cells surviving selection/screening can also be collected and used as substrates in subsequent rounds of recombination. For viruses that kill their host cells, recombinant genes resulting from recombination reside only in the progeny virus. If the screening or selective assay requires expression of recombinant genes in a cell, the IS recombinant genes should be transferred from the progeny virus to another vector, e.g., a plasmid vector, and retransfected into cells before selection/screening is performed.

For filamentous phage, the products of recombination are present in both cells surviving recombination and in phage extruded from these cells. The dual source of recombinant products provides some additional options relative to the plasmid-plasmid recombination. For example, DNA can be isolated from phage particles for use in a round of *in vitro* recombination. Alternatively, the progeny 2S phage can be used to transfect or infect cells surviving a previous round of screening/selection, or fresh cells transfected with fresh substrates for recombination.

8.6.1.2.2.3 VIRUS-VIRUS RECOMBINATION

The principles described for plasmid-plasmid and plasmid-viral recombination can be applied to virus-virus recombination with a few modifications. The initial substrates for recombination are cloned into a viral vector. Usually, the same vector is used for all substrates.

Preferably, the virus is one that, naturally or as a result of mutation, does not kill cells. After insertion, some viral genomes can be packaged in vitro or using a packaging cell line. The packaged viruses are used to infect cells at high multiplicity such that there is a high probability that a cell will receive multiple viruses bearing different substrates.

by obtaining the plasmid- borne substrates from a different species than that of the cells bearing the chromosome.

The plasmids bearing substrates for recombination are transfected into cells having chromosomal gene(s) to be evolved. Evolution can occur simply by propagating the culture, and can be accelerated by transferring plasmids between cells by conjugation or electroporation. Evolution can be further accelerated by use of mutator host cells or by seeding a culture of nonmutator host cells being evolved with mutator host cells and inducing intercellular transfer of plasmids by electroporation or conjugation. Preferably, mutator host cells used for seeding contain a negative selectable marker to facilitate isolation of a pure culture of the nonmutator cells being evolved. Selection/screening identifies cells bearing chromosomes and/or plasmids that have evolved toward acquisition of a desired function.

8.6.1.2.2.5 VIRUS-CHROMOSOME RECOMBINATION

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before screening, the ability to screen the "genetic potential" of the starting population is limited. For this reason, the rate of in vivo and whole genome stochastic &/or non-stochastic mutagenesis mediated cellular evolution would be facilitated by effecting poolwise recombination. Two strategies for poolwise recombination are described below (protoplast fusion and transduction).

8.6.1.2.2.7 PROTOPLAST FUSION

Protoplast fusion (discussed supra) mediated whole genome stochastic &/or non-stochastic mutagenesis is one format that can directly effect poolwise recombination. Whole gene stochastic &/or non-stochastic mutagenesis is the recursive recombination of whole genomes, in the form of one or more nucleic acid molecule(s) (fragments, chromosomes, episomes, etc), from a population of organisms, resulting in the production of new organisms having distributed genetic information from at least two of the starting population of organisms. The process of protoplast fusion is further illustrated in herein.

Progeny resulting from the fusion of multiple parent protoplasts have been observed (Hopwood & Wright, 1978), however, these progeny are rare (10^{-4} - 10^{-6}). The low frequency is attributed to the distribution of fusants arising from two, three, four, etc parents and the likelihood of the multiple recombination events (6 crossovers for a four parent cross) that would have to occur for multiparent progeny to arise. Thus, it is useful to enrich for the multiparent progeny. This can be accomplished, e.g., by repetitive fusion or enrichment for multiply fused protoplasts. The process of poolwise fusion and recombination is further illustrated herein.

8.6.1.2.2.8 REPETITIVE FUSION

Protoplasts of identified parental cells are prepared, fused and regenerated. Protoplasts of the regenerated progeny are then, without screening or enrichment, formed, fused and regenerated. This can be carried out for two, three, or more cycles before screening to increase the representation of multiparent progeny. The number of possible mutations/progeny doubles for each cycle. For example, if one cross produces predominantly progeny with 0, 1, and 2 mutations, a breeding of this population with itself

will produce progeny with 0, 1, 2, 3, and 4 mutations, the third cross up to eight, etc. The representation of the multiparent progeny from these subsequent crosses will not be as high as the single and double parent progeny, but it will be detectable and much higher than from a single cross. The repetitive fusion prior to screening is analogous to many sexual crosses within a population, and the individual thermal cycles of in vitro DNA stochastic &/or non-stochastic mutagenesis described supra. A factor effecting the value of this approach is the starting size of the parental population. As the population grows, it becomes more likely that a multiparent fusion will arise from repetitive fusions. For example, if 4 parents are fused twice, the 4 parent progeny will make up approximately 0.2% of the total progeny. This is sufficient to find in a population of 3000 (95% confidence), but better representation is preferable. If ten parents are fused twice >20% of the progeny will be four parent offspring.

8.6.1.2.2.9 ENRICHMENT FOR MULTIPLE FUSED PROTOPLASTS

After the fusion of a population of protoplasts, the fusants are typically diluted into hypotonic medium, to dilute out the fusing agent (e.g., 50% PEG). The fused cells can be grown for a short period to regenerate cell walls or separated directly and are then separated on the basis of size. This is carried out, e.g., by cell sorting, using light dispersion as an estimate of size, to isolate the largest fusants. Alternatively the fusants can be sorted by FACS on the basis of DNA content. The large fusants or those containing more DNA result from the fusion of multiple parents and are more likely to segregate to multiparent progeny. The enriched fusants are regenerated and screened directly or the progeny are fused recursively as above to further enrich the population for diverse mutant combinations.

8.6.1.2.2.10 TRANSDUCTION

Transduction can theoretically effect poolwise recombination, if the transducing phage particles contain predominantly host genomic DNA rather than phage DNA. If phage DNA is overly represented, then most cells will receive at least one undesired phage genome.

Phage particles generated from locked-in-prophage (supra) are useful for this purpose. A population of cells is infected with an appropriate transducing phage, and the lysate is collected and used to infect the same starting population. A high multiplicity of infection is employed to deliver multiple genomic fragments to each infected cell, thereby increasing the chance of producing recombinants containing mutations from more than two parent genomes.

The resulting transductants are recovered under conditions where phage can not propagate e.g., in the presence of citrate. This population is then screened directly or infected again with phage, with the resulting transducing particles being used to transduce the first progeny. This would mimic recursive protoplast fusion, multiple sexual recombination, and in vitro DNA stochastic &/or non-stochastic mutagenesis.

8.6.1.2.2.11 METHODS FOR WHOLE GENOME STOCHASTIC &/OR NON-STOCHASTIC MUTAGENESIS BY BLIND FAMILY STOCHASTIC &/OR NON-STOCHASTIC MUTAGENESIS OF PARSED GENOMES AND RECURSIVE CYCLES OF FORCED INTEGRATION AND EXCISION BY HOMOLOGOUS RECOMBINATION, AND SCREENING FOR IMPROVED PHENOTYPES

In vitro methods have been developed to reassemble single genes and operons, as set forth, e.g., herein. "Family" stochastic &/or non-stochastic mutagenesis of homologous genes within species and from different species is also an effective methods for accelerating molecular evolution. This section describes additional methods for extending these methods such that they can be applied to whole genomes.

In some cases, the genes that encode rate limiting steps in a biochemical process, or that contribute to a phenotype of interest are known. This method can be used to target family stochastic &/or non-stochastic mutagenized libraries to such loci, generating libraries of organisms with high quality family stochastic &/or non-stochastic mutagenized libraries of alleles at the locus of interest. An example of such a gene would be the evolution of a host chaperonin to more efficiently chaperone the folding of an overexpressed protein in *E. coli*.

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The goals of this process are to reassemble homologous genes from two or more species and to then integrate the stochastic &/or non-stochastic mutagenized genes into the chromosome of a target organism.

Integration of multiple stochastic &/or non-stochastic mutagenized genes at multiple loci can be achieved using recursive cycles of integration (generating duplications), excision (leaving the improved allele in the chromosome) and transfer of additional evolved genes by serially applying the same procedure.

In the first step, genes to be stochastic &/or non-stochastic mutagenized into suitable bacterial vectors are subcloned. These vectors can be plasmids, cosmids, BACS or the like. Thus, fragments from 100 bp to 100 kb can be handled. Homologous fragments are then "family stochastic &/or non-stochastic mutagenized" together (i.e. homologous fragments from different species or chromosomal locations are homologously recombined). As a simple case, homologs from two species (say, *E. coli* and *Salmonella*) are cloned, family stochastic &/or non-stochastic mutagenized in vitro and cloned into an allele replacement vector (e.g., a vector with a positively selectable marker, a negatively selectable marker and conditionally active origin of replication). The basic strategy for whole genome family stochastic &/or non-stochastic mutagenesis of parsed (subcloned) genomes is additionally set forth herein.

The vectors are transfected into *E. coli* and selected, e.g., for drug resistance. Most drug resistant cells should arise by homologous recombination between a family stochastic &/or non-stochastic mutagenized insert and a chromosomal copy of the cloned insert. Colonies with improved phenotype are screened (e.g., by mass spectroscopy for enzyme activity or small molecule production, or a chromogenic screen, or the like, depending on the phenotype to be assayed). Negative selection (i.e. sue selection) is imposed to force excision of tandem duplication. Roughly half C, of the colonies should retain the improved phenotype. Importantly, this process regenerates a "clean" chromosome in which the wild type locus is replaced with a family stochastic &/or non-stochastic mutagenized fragment

that encodes a beneficial allele. Since the chromosome is "clean" (i.e., has no vector sequences), other improved alleles can also be moved into this point on the chromosome by homologous recombination.

Selection or screening for improved phenotype can occur either after step 3 or step 4. If selection or screening takes place after step 3, then the improved allele can be conveniently moved to other strains by, for example, P I transduction. One can then regenerate a strain containing the improved allele but lacking vector sequences by "negative selection" against the suc marker. In subsequent rounds, independently identified improved variants of the gene can be sequentially moved into the improved strain (e.g., by P I transduction of the drug marked tandem duplication above). Transductants are screened for further improvement in phenotype by virtue of receiving the transduced tandem duplication, which itself contains the family stochastic &/or non-stochastic mutagenized genetic material. Negative selection is again imposed and the process of stochastic &/or non-stochastic mutagenesis the improved strain is recursively repeated as desired.

Although this process was described with reference to targeting a gene or genes of interest, it can be used "blindly," making no assumptions about which locus is to be targeted. This procedure is set forth herein. For example, the whole genome of an organism of interest is cloned into manageable fragments (e.g., 10 kb for plasmid-based methods). Homologous fragments are then isolated from related species. Forced recombination with chromosomal homologs creates chimeras.

8.6.1.2.2.12 METHODS FOR HIGH THROUGHPUT FAMILY STOCHASTIC &/OR NON-STOCHASTIC MUTAGENESIS OF GENES

For *E. coli*., cloning the genome in 10 kb fragments requires about 300 clones. The homologous fragments are isolated, e.g., from *Salmonella*. This gives roughly three hundred pairs of homologous fragments. Each pair is family stochastic &/or non-stochastic mutagenized and the stochastic &/or non-stochastic mutagenized fragments are cloned into an allele replacement vector. The inserts are integrated into the *E. coli* genome

The homologous DNA is used in a family stochastic &/or non-stochastic mutagenesis reaction for improvement of the desired function. Stochastic &/or non-stochastic mutagenesis the E. coli chaperonin gene DnaJ with other homologs is described below as an example. The example can be generalized to any other gene, including eukaryotic genes such as plant or animal genes (including mammalian genes), by following the format described.

As a first step, the E. coli DnaJ gene is cloned into an M13 phagemid vector. ssDNA is then produced, preferably in a dut(-) ung(-) strain so that Kunkel site directed mutagenesis protocols can be applied. Genomic DNA is then isolated from a non- E. coli source, such as Salmonella and Yersinia Pestis. The bacterial genomic DNAs are denatured and reannealed to the phagemid ssDNA (e.g., about 1 microgram of ssDNA). The reannealed product is treated with an enzyme such as Mung Bean nuclease that degrades ssDNA as an exonuclease but not as an endonuclease (the nuclease does not degrade mismatched DNA that is embedded in a larger annealed fragment). The standard Kunkel site directed mutagenesis protocol is used to extend the fragment and the target cells are transformed with the resulting mutagenized DNA.

In a first variation on the above, the procedure is adapted to the situation where the target gene or genes of interest are unknown. In this variation, the whole genome of the organism of interest is cloned in fragments (e.g., of about 10 kb each) into a phagemid. Single stranded phagemid DNA is then produced. Genomic DNA from the related species is denatured and annealed to the phagemids. Mung bean nuclease is used to trim away unhybridized DNA ends. Polymerase plus ligase is used to fill in the resulting gapped circles.

These clones are transformed into a mismatch repair deficient strain. When the mismatched molecules are replicated in the bacteria, most colonies contain both the E. coli and the homologous fragment. The two homologous genes are then isolated from the

colonies (e.g., either by standard plasmid purification or colony PCR) and stochastic &/or non-stochastic mutagenized.

Another approach to generating chimeras that requires no in vitro stochastic &/or non-stochastic mutagenesis is simply to clone the Salmonella genome into an allele replacement vector, transform E. coli, and select for chromosomal integrants. Homologous recombination between Salmonella genes and E. coli homologs generate stochastic &/or non-stochastic mutagenized chimeras. A global screen is done to screen for improved phenotypes. Alternately, recursive transformation and recombination is performed to increase diversity prior to screening. If colonies with improved phenotypes are obtained, it is verified that the improvement is due to allele replacement by P I transduction into a fresh strain and counterscreening for improved phenotype. A collection of such improved alleles can then be combined into one strain using the methods for whole genome stochastic &/or non-stochastic mutagenesis by blind family stochastic &/or non-stochastic mutagenesis of parsed genomes as set forth herein. Additionally, once these loci are identified, it is likely that further rounds of stochastic &/or non-stochastic mutagenesis and screening will yield further improvements. This could be done by cloning the chimeric gene and then using the methods described in this disclosure to breed the gene with homologs from many different strains of bacteria.

In general, the transformants contain clones of the homologue of the target gene (e.g., E. coli DnaJ in the example above). Mismatch repair in vivo results in a decrease in diversity of the gene. There are at least two solutions to this. First, transduction can be performed into a mismatch repair deficient strain. Alternatively or in addition, the M13 template DNA can be selectively degraded, leaving the cloned homologue. This can be done using methods similar to the standard Eckstein site directed mutagenesis technique (General texts which describe general molecular biological techniques useful herein, including mutagenesis, include Sambrook et al., Molecular Cloning - A Laboratory Manual (2nd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989 ("Sambrook") and Current Protocols in Molecular Biology, F.M. Ausubel et

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An analogous procedure is optionally performed in a PCR format. As applied to the DnaJ illustration above, DnaJ DNA is amplified by PCR with primers that build 30-mer priming sites on each end. The PCR is denatured and annealed with an excess of *Salmonella* genomic DNA. The *Salmonella* DnaJ gene hybridizes with the *E. coli* homologue. After treatment with Mung Bean nuclease, the resulting mismatched hybrid is PCR amplified with the flanking 30-mer primers. This PCR product can be used directly for family stochastic &/or non-stochastic mutagenesis. As genomics provides an increasing amount of sequence information, it is increasingly possible to directly PCR amplify homologs with designed primers. For example, given the sequence of the *E. coli* genome and of a related genome (i.e. *Salmonella*), each genome can be PCR amplified with designed primers in, e.g., 5 kb fragments. The homologous fragments can be put together in a pairwise fashion for stochastic &/or non-stochastic mutagenesis. For genome stochastic &/or non-stochastic mutagenesis, the stochastic &/or non-stochastic mutagenized products are cloned into the allele replacement vector and bred into the genome as described supra.

8.6.1.2.2.13 HYPER-RECOMBINOGENIC RecA CLONES

The invention further provides hyper-recombinogenic RecA proteins (see, the examples below). It is fully expected that one of skill can make a variety of related recombinogenic proteins given the disclosed sequences.

It will be appreciated that conservative substitutions of the given sequences can be used to produce nucleic acids which encode hyperrecombinogenic clones. "Conservatively modified variations" of a particular nucleic acid sequence refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic

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acids with highly similar properties, are also readily identified as being highly similar to a disclosed construct. Such conservatively substituted variations of each explicitly disclosed sequence are a feature of the present invention.

Nucleic acids which hybridize under stringent conditions to the nucleic acids in the figures are a feature of the invention. "Stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and northern hybridizations are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes part I chapter 2 "overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5C lower than the thermal melting point (T.) for the specific sequence at a defined ionic strength and pH. The T. is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T. for a particular probe. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization.

Nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

Finally, preferred nucleic acids encode hyper-recombinogenic RecA proteins which are at least one order of magnitude (10 times) as active as a wild- type RecA protein in a standard assay for Rec A activity.



1. The first part of the document is a list of references. The references are listed in a standard format, with the author's name, the title of the work, and the publisher. The references are as follows:

1. J. H. Van der Linde, *Die Geschiedenis van die Kaapkolonie*, 1898, 1900, 1902, 1904, 1906, 1908, 1910, 1912, 1914, 1916, 1918, 1920, 1922, 1924, 1926, 1928, 1930, 1932, 1934, 1936, 1938, 1940, 1942, 1944, 1946, 1948, 1950, 1952, 1954, 1956, 1958, 1960, 1962, 1964, 1966, 1968, 1970, 1972, 1974, 1976, 1978, 1980, 1982, 1984, 1986, 1988, 1990, 1992, 1994, 1996, 1998, 2000, 2002, 2004, 2006, 2008, 2010, 2012, 2014, 2016, 2018, 2020, 2022, 2024, 2026, 2028, 2030, 2032, 2034, 2036, 2038, 2040, 2042, 2044, 2046, 2048, 2050, 2052, 2054, 2056, 2058, 2060, 2062, 2064, 2066, 2068, 2070, 2072, 2074, 2076, 2078, 2080, 2082, 2084, 2086, 2088, 2090, 2092, 2094, 2096, 2098, 2100, 2102, 2104, 2106, 2108, 2110, 2112, 2114, 2116, 2118, 2120, 2122, 2124, 2126, 2128, 2130, 2132, 2134, 2136, 2138, 2140, 2142, 2144, 2146, 2148, 2150, 2152, 2154, 2156, 2158, 2160, 2162, 2164, 2166, 2168, 2170, 2172, 2174, 2176, 2178, 2180, 2182, 2184, 2186, 2188, 2190, 2192, 2194, 2196, 2198, 2200, 2202, 2204, 2206, 2208, 2210, 2212, 2214, 2216, 2218, 2220, 2222, 2224, 2226, 2228, 2230, 2232, 2234, 2236, 2238, 2240, 2242, 2244, 2246, 2248, 2250, 2252, 2254, 2256, 2258, 2260, 2262, 2264, 2266, 2268, 2270, 2272, 2274, 2276, 2278, 2280, 2282, 2284, 2286, 2288, 2290, 2292, 2294, 2296, 2298, 2300, 2302, 2304, 2306, 2308, 2310, 2312, 2314, 2316, 2318, 2320, 2322, 2324, 2326, 2328, 2330, 2332, 2334, 2336, 2338, 2340, 2342, 2344, 2346, 2348, 2350, 2352, 2354, 2356, 2358, 2360, 2362, 2364, 2366, 2368, 2370, 2372, 2374, 2376, 2378, 2380, 2382, 2384, 2386, 2388, 2390, 2392, 2394, 2396, 2398, 2400, 2402, 2404, 2406, 2408, 2410, 2412, 2414, 2416, 2418, 2420, 2422, 2424, 2426, 2428, 2430, 2432, 2434, 2436, 2438, 2440, 2442, 2444, 2446, 2448, 2450, 2452, 2454, 2456, 2458, 2460, 2462, 2464, 2466, 2468, 2470, 2472, 2474, 2476, 2478, 2480, 2482, 2484, 2486, 2488, 2490, 2492, 2494, 2496, 2498, 2500, 2502, 2504, 2506, 2508, 2510, 2512, 2514, 2516, 2518, 2520, 2522, 2524, 2526, 2528, 2530, 2532, 2534, 2536, 2538, 2540, 2542, 2544, 2546, 2548, 2550, 2552, 2554, 2556, 2558, 2560, 2562, 2564, 2566, 2568, 2570, 2572, 2574, 2576, 2578, 2580, 2582, 2584, 2586, 2588, 2590, 2592, 2594, 2596, 2598, 2600, 2602, 2604, 2606, 2608, 2610, 2612, 2614, 2616, 2618, 2620, 2622, 2624, 2626, 2628, 2630, 2632, 2634, 2636, 2638, 2640, 2642, 2644, 2646, 2648, 2650, 2652, 2654, 2656, 2658, 2660, 2662, 2664, 2666, 2668, 2670, 2672, 2674, 2676, 2678, 2680, 2682, 2684, 2686, 2688, 2690, 2692, 2694, 2696, 2698, 2700, 2702, 2704, 2706, 2708, 2710, 2712, 2714, 2716, 2718, 2720, 2722, 2724, 2726, 2728, 2730, 2732, 2734, 2736, 2738, 2740, 2742, 2744, 2746, 2748, 2750, 2752, 2754, 2756, 2758, 2760, 2762, 2764, 2766, 2768, 2770, 2772, 2774, 2776, 2778, 2780, 2782, 2784, 2786, 2788, 2790, 2792, 2794, 2796, 2798, 2800, 2802, 2804, 2806, 2808, 2810, 2812, 2814, 2816, 2818, 2820, 2822, 2824, 2826, 2828, 2830, 2832, 2834, 2836, 2838, 2840, 2842, 2844, 2846, 2848, 2850, 2852, 2854, 2856, 2858, 2860, 2862, 2864, 2866, 2868, 2870, 2872, 2874, 2876, 2878, 2880, 2882, 2884, 2886, 2888, 2890, 2892, 2894, 2896, 2898, 2900, 2902, 2904, 2906, 2908, 2910, 2912, 2914, 2916, 2918, 2920, 2922, 2924, 2926, 2928, 2930, 2932, 2934, 2936, 2938, 2940, 2942, 2944, 2946, 2948, 2950, 2952, 2954, 2956, 2958, 2960, 2962, 2964, 2966, 2968, 2970, 2972, 2974, 2976, 2978, 2980, 2982, 2984, 2986, 2988, 2990, 2992, 2994, 2996, 2998, 3000, 3002, 3004, 3006, 3008, 3010, 3012, 3014, 3016, 3018, 3020, 3022, 3024, 3026, 3028, 3030, 3032, 3034, 3036, 3038, 3040, 3042, 3044, 3046, 3048, 3050, 3052, 3054, 3056, 3058, 3060, 3062, 3064, 3066, 3068, 3070, 3072, 3074, 3076, 3078, 3080, 3082, 3084, 3086, 3088, 3090, 3092, 3094, 3096, 3098, 3100, 3102, 3104, 3106, 3108, 3110, 3112, 3114, 3116, 3118, 3120, 3122, 3124, 3126, 3128, 3130, 3132, 3134, 3136, 3138, 3140, 3142, 3144, 3146, 3148, 3150, 3152, 3154, 3156, 3158, 3160, 3162, 3164, 3166, 3168, 3170, 3172, 3174, 3176, 3178, 3180, 3182, 3184, 3186, 3188, 3190, 3192, 3194, 3196, 3198, 3200, 3202, 3204, 3206, 3208, 3210, 3212, 3214, 3216, 3218, 3220, 3222, 3224, 3226, 3228, 3230, 3232

A plasmid containing *recA* and *recE* genes is stochastic &/or non-stochastic mutagenized (either using these genes as single starting points, or by family stochastic &/or non-stochastic mutagenesis (with for example *red⁺* and *red⁻*, or other homologous genes identified from available sequence databases). This stochastic &/or non-stochastic mutagenized library is then cloned into a vector with a selectable marker and transformed into an appropriate recombination-deficient strain. The library of cells would then be transformed with a second selectable marker, either borne on a suicide vector or as a linear DNA fragment with regions at its ends that are homologous to a target sequence (either in the plasmid or in the host chromosome). Integration of this marker by homologous recombination is a selectable event, dependent on the activity of the *recE* and *recT* gene products. The *recE* / *recT* genes are isolated from cells in which homologous recombination has occurred. The process is repeated several times to enrich for the most efficient variants before the next round of stochastic &/or non-stochastic mutagenesis is performed. In addition, cycles of recombination without selection can be performed to increase the diversity of a cell population prior to selection.

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Described below is a method used to reassemble four strains of *Streptomyces coelicolor*. From the initial four strains each containing a unique nutritional marker, three to four rounds of recursive pooled protoplast fusion was sufficient to generate a population of stochastic &/or non-stochastic mutagenized organisms containing all 16 possible combinations of the four markers. This represents a 10^6 fold improvement in the generation of four parent progeny as compared to a single pooled fusion of the four strains.

Protoplasts were generated from several strains of *S. coelicolor*, pooled and fused. Mycelia were regenerated and allowed to sporulate. The spores were collected, allowed to grow into Mycelia, formed into protoplasts, pooled and fused and the process repeated for three to four rounds. the resulting spores were then subject to screening.

The basic protocol for generating a whole genome stochastic &/or non-stochastic mutagenized library from four *S. coelicolor* strains, each having one of four distinct markers, was as follows. Four mycelial cultures, each of a strain having one of four different markers, were grown to early stationary phase. The mycelia from each were harvested by centrifugation and washed. Protoplasts from each culture were prepared as follows. Approximately 10^9 *S. coelicolor* spores were inoculated into 50ml YEME with 0.5% Glycine in a 250ml baffled flask. The spores were incubated at 30C for 36-40 hours in an orbital shaker. Mycelium were verified using a microscope. Some strains needed an additional day of growth. The culture was transferred into a 50ml tube and centrifuged at 4,000 rpm for 10 min. The mycelium were twice washed with 10.3% sucrose and centrifuged at 4,000 rpm for 10 min. (mycelium can be stored at about 80C after wash). 5ml of lysozyme was added to the about 0.5g of mycelium pellet. The pellet was suspended and incubated at 30C for 20-60 min., with gentle shaking every 10 min. The microscope was checked for protoplasting every 20 min. Once the majority were protoplasts, protoplasting was stopped by adding 10ml of P buffer. The protoplasts were filtered through cotton and the protoplast spun down at 3,000rpm for 7 min at room temperature. The supernatant was discarded and the protoplast gently resuspended, adding a suitable amount of P buffer according to the pellet size (usually about 50OW). Ten-fold

serial dilutions were made in P buffer, and the protoplasts counted at a 10^{-2} dilution. Protoplasts were adjusted to 10^{10} protoplasts per ml.

The protoplasts from each culture were quantitated by microscopy. 10^8 protoplast from each culture were mixed in the same tube, washed, and then fused by the addition of 50% PEG. The fused protoplasts were diluted and plated regeneration medium and incubated until the colonies were sporulating (four days). Spores were harvested and washed. These spores represent a pool of all the recombinants and parents form the fusion.

A sample of the pooled spores was then used to inoculate a single liquid culture. The culture was grown to early stationary phase, the mycelia harvested, and protoplasts prepared. 10^8 protoplasts from this "mycelial library" were then fused with themselves by the addition of 50%PEG. The protoplast fusion/regeneration/harvesting/protoplast preparation steps were repeated two times. The spores resulting from the fourth round of fusion were considered the "whole genome stochastic &/or non-stochastic mutagenized library" and they were screened for the frequency of the 16 possible combinations of the four markers

In particular, adding rounds of recombination prior to selection produced significant increases in the number of clones which incorporated all four of the relevant selectable markers, indicating that the population became increasingly diverse by recursive pooling and sporulation.

The four strains of the four parent stochastic &/or non-stochastic mutagenesis were each auxotrophic for three and prototrophic for one of four possible nutritional markers: arginine (A), cystine (C), proline (P), and/or uracil (U). Spores from each fusion were plated in each of the 16 possible combinations of these four nutrients, and the percent of the population growing on a particulate medium was calculated as the ration of those colonies from a selective plate to those growing on a plate having all four nutrients (all variants grow on the medium having all four nutrients, thus the colonies from this plate thus represent the total viable population). The corrected percentages for each of the no,

8.7.6.1 EXEMPLARY PROCEDURE FOR YEAST PROTOPLASTING

Protoplast preparation in yeast is reviewed by Morgan, in *Protoplasts* (Birkhauser Verlag, Basel, 1983). Fresh cells ($\sim 10^8$) are washed with buffer, for example 0.1 M potassium phosphate, then resuspended in this same buffer containing a reducing agent, such as 50 mM DTT, incubated for 1 h at 30°C with gentle agitation, and then washed again with buffer to remove the reducing agent. These cells are then resuspended in buffer containing a cell wall degrading enzyme, such as Novozyme 234 (1 mg/mL), and any of a variety of osmotic stabilizers, such as sucrose, sorbitol, NaCl, KCl, $MgSO_4$, $MgCl_2$, or NH_4Cl at any of a variety of concentrations. These suspensions are then incubated at 30°C with gentle shaking (~ 60 rpm) until protoplasts are released. To generate protoplasts that are more likely to produce productive fusants several strategies are possible.

Protoplast formation can be increased if the cell cycle of the protoplasts have been synchronized to be halted at G1. In the case of *S. cerevisiae* this can be accomplished by the addition of mating factors, either α or a (Curran & Carter, *J Gen. Microbiol.* 129, 1589-1591 (1983)). These peptides act as adenylate cyclase inhibitors which by decreasing the cellular level of cAMP arrest the cell cycle at G1. In addition, sex factors have been shown to induce the weakening of the cell wall in preparation for the sexual fusion of α and a cells (Crandall & Brock, *Bacteriol. Rev.* 32, 139-163 (1968); Osumi et al., *Arch. Microbiol.* 97, 27-38 (1974)). Thus in the preparation of protoplasts, cells can be treated with mating factors or other known inhibitors of adenylate cyclase, such as leflunomide or the killer toxin from *K. lactis*, to arrest them at G1 (Sugisaki et al., *Nature* 304, 464-466 (1983)). Then after fusing of the protoplasts (step 2), cAMP can be added to the regeneration medium to induce S-phase and DNA synthesis. Alternatively, yeast strains having a temperature sensitive mutation in the CDC4 gene can be used, such that cells could be synchronized and arrested at G1. After fusion cells are returned to the permissive temperature so that DNA synthesis and growth resumes.

Once suitable protoplasts have been prepared, it is necessary to induce fusion by physical or chemical means. An equal number of protoplasts of each cell type is mixed in

expressed from naturally associated regulatory sequences within yeast. However, alternatively, individual genes can be linked to yeast regulatory elements to form an expression cassette, and a concatemer of such cassettes, each containing a different gene, can be inserted into a YAC.

In some instances, fragments are incorporated into the yeast genome, and stochastic &/or non-stochastic mutagenesis is used to evolve improved yeast strains. In other instances, fragments remain as components of YACs throughout the stochastic &/or non-stochastic mutagenesis process, and after acquisition of a desired property, the YACs are transferred to a desired recipient cell.

8.7.8 STOCHASTIC &/OR NON-STOCHASTIC MUTAGENESIS OF GENES FOR BIOREMEDIATION

Modern industry generates many pollutants for which the environment can no longer be considered an infinite sink. Naturally occurring microorganisms are able to metabolize thousands of organic compounds, including many not found in nature (e.g xenobiotics). Bioremediation, the deliberate use of microorganisms for the biodegradation of man-made wastes, is an emerging technology that offers cost and practicality advantages over traditional methods of disposal. The success of bioremediation depends on the availability of organisms that are able to detoxify or mineralize pollutants.

Microorganisms capable of degrading specific pollutants can be generated by genetic engineering and recursive sequence recombination. Although bioremediation is an aspect of pollution control, a more useful approach in the long term is one of prevention before industrial waste is pumped into the environment. Exposure of industrial waste streams to recursive sequence recombination-generated microorganisms capable of degrading the pollutants they contain would result in detoxification of mineralization of these pollutants before the waste stream enters the environment. Issues of releasing recombinant organisms can be avoided by containing them within bioreactors fitted to the industrial effluent pipes. This approach would also allow the microbial mixture used to be

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adjusted to best degrade the particular wastes being produced. Finally, this method would avoid the problems of adapting to the outside world and dealing with competition that face many laboratory microorganisms.

In the wild, microorganisms have evolved new catabolic activities enabling them to exploit pollutants as nutrient sources for which there is no competition. However, pollutants that are present at low concentrations in the environment may not provide a sufficient advantage to stimulate the evolution of catabolic enzymes. For a review of such naturally occurring evolution of biodegradative pathways and the manipulation of some of microorganisms by classical techniques, see Ramos et al., *Bio/Technology* 12:1349-1355 (1994).

Generation of new catabolic enzymes or pathways for bioremediation has thus relied upon deliberate transfer of specific genes between organisms (Wackett et al., *supra*), forced matings between bacteria with specific catabolic capabilities (Brenner et al. *Biodegradation* 5:359-377 (1994)), or prolonged selection in a chemostat. Some researchers have attempted to facilitate evolution via naturally occurring genetic mechanisms in their chemostat selections by including microorganisms with a variety of catabolic pathways (Kellogg et al. *Science* 214:1133-1135 (1981); Chakrabarty *American Society of Micro. Biol. News* 62:130-137 (1996)). For a review of efforts in this area, see Cameron et al. *Applied Biochem. Biotech* 38:105-140 (1993).

Current efforts in improving organisms for bioremediation take a labor-intensive approach in which many parameters are optimized independently, including transcription efficiency from native and heterologous promoters, regulatory circuits and translational efficiency as well as improvement of protein stability and activity (Timmis et al. *Ann. Rev. Microbiol.* 48:525- 527 (1994)).

A recursive sequence recombination approach overcomes a number of limitations in the bioremediation capabilities of naturally occurring microorganisms. Both enzyme

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DOUGLAS B. COOPER

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BOOK REVIEW

8.7.8.3 POLYCHLORINATED BIPHENYLS AND POLYCYCLIC AROMATIC HYDROCARBONS

Polychlorinated Biphenyls (PCBs) and Polycyclic Aromatic Hydrocarbons (PAHs) are families of structurally related compounds that are major pollutants at many Superfund sites. Bacteria transformed with plasmids encoding enzymes with broader substrate specificity have been used commercially. In nature, no known pathways have been generated in a single host that degrade the larger PAHs or more heavily chlorinated PCBs. Indeed, often the collaboration of anaerobic and aerobic bacteria are required for

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Substrate specificity in the PCB pathway largely results from enzymes involved in initial dioxygenation reactions, and can be significantly altered by mutations in those enzymes (Erickson et al. *Applied Environ. Micro.* 59:3858-38662 (1993); Furukawa et al. *J. Bact.* 175:5224-5232 (1993)). Mineralization of PAHs and PCBs requires that the downstream pathway is able to metabolize the products of the initial reaction (Brenner et al. *Biodegradation* 5:359-377 (1994)). In this case, recursive sequence recombination of the entire pathway with selection for bacteria able to use the PCE or PAH as the sole carbon source will allow production of novel PCB and PAH degrading bacteria.

A general method for evolving genes for the catabolism of insoluble herbicides is exemplified as follows for atrazine. Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine] is a moderately persistent herbicide which is frequently detected in ground and surface water at concentrations exceeding the 3 ppb health advisory level set by the EPA. Atrazine can be slowly metabolized by a *Pseudomonas* species (Mandelbaum et al. Appl. Environ. Micro. 61:1451-1457 (1995)). The enzymes catalyzing the first two steps in atrazine metabolism by *Pseudomonas* are encoded by genes *AtzA* and *AtzB* (de Souza et al. Appl. Environ. Micro. 61:3373-3378 (1995)). These genes have been cloned in a 6.8 kb fragment into pUC18 (*AtzAB*-pUC). *E. coli* carrying

this plasmid converts atrazine to much more soluble metabolites. It is thus possible to screen for enzyme activity by growing bacteria on plates containing atrazine. The herbicide forms an opaque precipitate in the plates, but cells containing AtzAB-pU18 secrete atrazine degrading enzymes, leading to a clear halo around those cells or colonies. Typically, the size of the halo and the rate of its formation can be used to assess the level of activity so that picking colonies with the largest halos allows selection of the more active or highly produced atrazine degrading enzymes.

Thus, the plasmids carrying these genes can be subjected to the recursive sequence recombination formats described above to optimize the catabolism of atrazine in *E. coli* or another host of choice, including *Pseudomonas*. After each round of recombination, screening of host colonies expressing the evolved genes can be done on agar plates containing atrazine to observe halo formation. This is a generally applicable method for screening enzymes that metabolize insoluble compounds to those that are soluble (e.g., polycyclic aromatic hydrocarbons). Additionally, catabolism of atrazine can provide a source of nitrogen for the cell; if no other nitrogen is available, cell growth will be limited by the rate at which the cells can catabolize nitrogen. Cells able to utilize atrazine as a nitrogen source can thus be selected from a background of non-utilizers or poor-utilizers.

Bacteria are used commercially to detoxify arsenate waste generated by the mining of arsenopyrite gold ores. As well as mining effluent, industrial waste water is often contaminated with heavy metals (e.g., those used in the manufacture of electronic components and plastics). Thus, simply to be able to perform other bioremedial functions, microorganisms must be resistant to the levels of heavy metals present, including mercury, arsenate, chromate, cadmium, silver, etc.

metal contamination can be effected in a number of ways including changing the solubility or bioavailability of the metal, changing its redox state (e.g. toxic mercuric chloride is detoxified by reduction to the much more volatile elemental mercury) and even by bioaccumulation of the metal by immobilized bacteria or is plants. The accumulation of metals to a sufficiently high concentration allows metal to be recycled; smelting burns off the organic part of the organism, leaving behind reusable accumulated metal. Resistances to a number of heavy metals (arsenate, cadmium, cobalt, chromium, copper, mercury, nickel, lead, silver, and zinc) are plasmid encoded in a number of species including *Staphylococcus* and *Pseudomonas* (Silver et al. *Environ. Health Perspect.* 102:107-113 (1994); Ji et al. *J. Ind. Micro.* 14:61-75 (1995). These genes also confer heavy metal resistance on other species as well (e.g., *E. coli*). The recursive sequence recombination techniques of the instant invention (RSR) can be used to increase microbial heavy metal tolerances, as well as to increase the extent to which cells will accumulate heavy metals. For example, the ability of *E. coli* to detoxify arsenate can be improved at least 100-fold by RSR.

Cyanide is very efficiently used to extract gold from rock containing as little as 0.2 oz per ton. This cyanide can be microbially neutralized and used as a nitrogen source by fungi or bacteria such as *Pseudomonas fluorescens*. A problem with microbial cyanide degradation is the presence of toxic heavy metals in the leachate. RSR can be used to increase the resistance of bioremedial microorganisms to toxic heavy metals, so that they will be able to survive the levels present in many industrial and Superfund sites. This will allow them to biodegrade organic pollutants including but not limited to aromatic hydrocarbons, halogenated hydrocarbons, and biocides.

8.7.8.6 MICROBIAL MINING

"Bioleaching" is the process by which microbes convert insoluble metal deposits (usually metal sulfides or oxides) into soluble metal sulfates. Bioleaching is commercially important in the mining of arsenopyrite, but has additional potential in the detoxification and recovery of metals and acids from waste dumps. Naturally occurring bacteria capable

of bioleaching are reviewed by Rawlings and Silver (Bio/Technology 13:773-778 (1995)).

These bacteria are typically divided into groups by their preferred temperatures for growth. The more important mesophiles are Thiobacillus and Leptospirillum species. Moderate thermophiles include Sulfobacillus species. Extreme thermophiles include Sulfolobus species. Many of these organisms are difficult to grow in commercial industrial settings, making their catabolic abilities attractive candidates for transfer to and optimization in other organisms such as Pseudomonas, Rhodococcus, T. ferrooxidans or E. coli. Genetic systems are available for at least one strain of T. ferrooxidans, allowing the manipulation of its genetic material on plasmids.

The recursive sequence recombination methods described above can be used to optimize the catalytic abilities in native hosts or heterologous hosts for evolved bioleaching genes or pathways, such as the ability to convert metals from insoluble to soluble salts. In addition, leach rates of particular ores can be improved as a result of, for example, increased resistance to toxic compounds in the ore concentrate, increased specificity for certain substrates, ability to use different substrates as nutrient sources, and so on.

8.7.8.7 OIL DESULFURIZATION

The presence of sulfur in fossil fuels has been correlated with corrosion of pipelines, pumping, and refining equipment, and with the premature breakdown of combustion engines. Sulfur also poisons many catalysts used in the refining of fossil fuels. The atmospheric emission of sulfur combustion products is known as acid rain.

Microbial desulfurization is an appealing bioremediation application. Several bacteria have been reported that are capable of catabolizing dibenzothiophene (DBT), which is the representative compound of the class of sulfur compounds found in fossil fuels. U.S. Patent No. 5,356,801 discloses the cloning of a DNA molecule from Rhodococcus rhodochrous capable of biocatalyzing the desulfurization of oil. Denome et

more specific, and able to more completely reduce (and thus detoxify) their target compounds (examples of which include but are not limited to nitrotoluenes and nitrobenzenes). Nitro-reductases can be isolated from bacteria isolated from explosive-contaminated soils, such as *Morganella morganii* and *Enterobacter cloacae* (Bryant et. al., 1991. J. Biol Chem. 266:4126-4130). A preferred selection method is to look for increased resistance to the organo-nitro compound of interest, since that will indicate that the enzyme is also able to reduce any toxic partial reduction products of the original compound.

8.7.8.9 ALTERNATIVE SUBSTRATES FOR CHEMICAL SYNTHESIS

Metabolic engineering can be used to alter microorganisms that produce industrially useful chemicals, so that they will grow using alternate and more abundant sources of nutrients, including human-produced industrial wastes. This typically involves providing both a transport system to get the alternative substrate into the engineered cells and catabolic enzymes from the natural host organisms to the engineered cells.

In some instances, enzymes can be secreted into the medium by engineered cells to degrade the alternate substrate into a form that can more readily be taken up by the engineered cells; in other instances, a batch of engineered cells can be grown on one preferred substrate, then lysed to liberate hydrolytic enzymes for the alternate substrate into the medium, while a second inoculum of the same engineered host or a second host is added to utilize the hydrolyzate.

The starting materials for recursive sequence recombination will typically be genes for utilization of a substrate or its transport. Examples of nutrient sources of interest include but are not limited to lactose, whey, galactose, mannitol, xylan, cellobiose, cellulose and sucrose, thus allowing cheaper production of compounds including but not limited to ethanol, tryptophan, rhamnolipid surfactants, xanthan gum, and polyhydroxylalkanoate. For a review of such substrates as desired target substances, see Cameron et al. (Appl. Biochem. Biotechnol. 38:105-140 (1993)). The recursive sequence recombination methods described above can be used to optimize the ability of native hosts

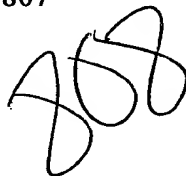
or heterologous hosts to utilize a substrate of interest, to evolve more efficient transport systems, to increase or alter specificity for certain substrates, and so on.

8.7.8.10 MODIFICATION OF CELL PROPERTIES

Although not strictly examples of manipulation of intermediary metabolism, recursive sequence recombination techniques can be used to improve or alter other aspects of cell properties, from growth rate to ability to secrete certain desired compounds to ability to tolerate increased temperature or other environmental stresses. Some examples of traits engineered by traditional methods include expression of heterologous proteins in bacteria, yeast, and other eukaryotic cells, antibiotic resistance, and phage resistance. Any of these traits is advantageously evolved by the recursive sequence recombination techniques of the instant invention. Examples include replacement of one nutrient uptake system (e.g. ammonia in *Methylophilus methylotrophus*) with another that is more energy efficient; expression of haemoglobin to improve growth under conditions of limiting oxygen; redirection of toxic metabolic end products to less toxic compounds; expression of genes conferring tolerance to salt, drought and toxic compounds and resistance to pathogens, antibiotics and bacteriophage, reviewed in Cameron et. al. *Appl Biochem Biotechnol*, 38:105-140 (1993).

The heterologous genes encoding these functions all have the potential for further optimization in their new hosts by existing recursive sequence recombination technology. Since these functions increase cell growth rates under the desired growth conditions, optimization of the genes by evolution simply involves recombining the DNA recursively and selecting the recombinants that grow faster with limiting oxygen, higher toxic compound concentration, or whatever is the appropriate growth condition for the parameter being improved.

Since these functions increase cell growth rates under the desired growth conditions, optimization of the genes by "evolution" can simply involve "stochastic &/or non-stochastic mutagenesis" the DNA and selecting the recombinants that grow faster with limiting oxygen, higher toxic compound concentration or whatever restrictive



condition is being overcome. Cultured mammalian cells also require essential amino acids to be present in the growth medium. This requirement could also be circumvented by expression of heterologous metabolic pathways that synthesize these amino acids (Rees et al., Biotechnology 8:629-633 (1990)). Recursive sequence recombination would provide a mechanism for optimizing the expression of these genes in mammalian cells. Once again, a preferred selection would be for cells that can grow in the absence of added amino acids.

Yet another candidate for improvement through the techniques of the invention is symbiotic nitrogen fixation. Genes involved in nodulation (nod, ndv), nitrogen reduction (nif, fix), host range determination (nod, hsp), bacteriocin production (tfx), surface polysaccharide synthesis (exo) and energy utilization (dct, hup) which have been identified (Paa, Biotech. Adv. 9:173-184 (1991)). The main function of recursive sequence recombination in this case is in improving the survival of strains that are already known to be better nitrogen fixers. These strains tend to be less good at competing with strains already present in the environment, even though they are better at nitrogen fixation. Targets for recursive sequence recombination such as nodulation and host range determination genes can be modified and selected for by their ability to grow on the new host.

Similarly any bacteriocin or energy utilization genes that will improve the competitiveness of the strain will also result in greater growth rates. Selection can simply be performed by subjecting the target genes to recursive sequence recombination and forcing the inoculant to compete with wild type nitrogen fixing bacteria. The better the nitrogen fixing bacteria grow in the new host, the more copies of their recombined genes will be present for the next round of recombination. This growth rate differentiating selection is described above in detail.

8.7.8.11 BIODETECTORS / BIOSENSORS

Bioluminescence or fluorescence genes can be used as reporters by fusing them to specific regulatory genes (Cameron et. al. Appl. Biochem Biotechnol, 38:105- 140 (1993)). A specific example is one in which the luciferase genes luxCDABE of *Vibrio*



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Recursive sequence recombination can also be used to alter the specificity of biosensors. The regulatory region, and transcriptional activators that interact with this region and with the chemicals that induce transcription can also be stochastic &/or non-stochastic mutagenized. This should generate regulatory systems in which transcription is activated by analogues of the normal inducer, so that biodetectors for different chemicals can be developed.

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evolved by recursive sequence recombination, such as hormones, growth factors, metals and drugs. These receptors may be intracellular and direct activators of transcription, or they may be membrane bound receptors that activate transcription of the signal indirectly, for example by a phosphorylation cascade. They may also not act on transcription at all, but may produce a signal by some post-transcriptional modification of a component of the signal generating pathway. These receptors may also be generated by fusing domains responsible for binding different ligands with different signaling domains. Again, recursive sequence recombination can be used to increase the amplitude of the signal generated to optimize expression and functioning of chimeric receptors, and to alter the specificity of the chemicals detected by the receptor.

8.8 PROMOTING GENETIC EXCHANGE

Some methods of the invention effect recombination of cellular DNA by propagating cells under conditions inducing exchange of DNA between cells. DNA exchange can be promoted by generally applicable methods such as electroporation, biolistics, cell fusion, or in some instances, by conjugation, transduction, or agrobacterium mediated transfer and meiosis. For example, Agrobacterium can transform *S. cerevisiae* with T-DNA, which is incorporated into the yeast genome by both homologous recombination and a gap repair mechanism. (Piers et al., Proc. Natl. Acad. Sci. USA 93(4),1613-8 (1996)).

In some methods, initial diversity between cells (i.e., before genome exchange) is induced by chemical or radiation-induced mutagenesis of a progenitor cell type, optionally followed by screening for a desired phenotype. In other methods, diversity is natural as where cells are obtained from different individuals, strains or species.

In some stochastic &/or non-stochastic mutagenesis methods, induced exchange of DNA is used as the sole means of effecting recombination in each cycle of recombination. In other methods, induced exchange is used in combination with natural sexual recombination of an organism. In other methods, induced exchange and/or natural sexual

recombination are used in combination with the introduction of a fragment library. Such a fragment library can be a whole genome, a whole chromosome, a group of functionally or genetically linked genes, a plasmid, a cosmid, a mitochondrial genome, a viral genome (replicative and nonreplicative) or specific or random fragments of any of these. The DNA can be linked to a vector or can be in free form. Some vectors contain sequences promoting homologous or nonhomologous recombination with the host genome. Some fragments contain double stranded breaks such as caused by shearing with glass beads, sonication, or chemical or enzymatic fragmentation, to stimulate recombination. In each case, DNA can be exchanged between cells after which it can undergo recombination to form hybrid genomes. Generally, cells are recursively subject to recombination to increase the diversity of the population prior to screening. Cells bearing hybrid genomes, e.g., generated after at least one, and usually several cycles of recombination are screened for a desired phenotype, and cells having this phenotype are isolated. These cells can additionally form starting materials for additional cycles of recombination in a recursive recombination/selection scheme.

8.8.1 PROTOPLAST FUSION

One means of promoting exchange of DNA between cells is by fusion of cells, such as by protoplast fusion. A protoplast results from the removal from a cell of its cell wall, leaving a membrane-bound cell that depends on an isotonic or hypertonic medium for maintaining its integrity. If the cell wall is partially removed, the resulting cell is strictly referred to as a spheroplast and if it is completely removed, as a protoplast. However, here the term protoplast includes spheroplasts unless otherwise indicated.

Protoplast fusion is described by Shaffner et al., Proc. Natl. Acad. Sci. USA 77, 2163 (1980) and other exemplary procedures are described by Yoakum et al., US 4,608,339, Takahashi et al., US 4,677,066 and Sambrooke et al., at Ch. 16. Protoplast fusion has been reported between strains, species, and genera (e.g., yeast and chicken erythrocyte). Protoplasts can be prepared for both bacterial and eukaryotic cells, including mammalian cells and plant cells, by several means including chemical treatment to strip

cell walls. For example, cell walls can be stripped by digestion with a cell wall degrading enzyme such as lysozyme in a 10-20% sucrose, 50 mM EDTA buffer. Conversion of cells to spherical protoplasts can be monitored by phase-contrast microscopy. Protoplasts can also be prepared by propagation of cells in media supplemented with an inhibitor of cell wall synthesis, or use of mutant strains lacking capacity for cell wall formation. Preferably, eukaryotic cells are synchronized in G1 phase by arrest with inhibitors such as - factor, K. lactis killer toxin, leflonamide and adenylate cyclase inhibitors. Optionally, some but not all, protoplasts to be fused can be killed and/or have their DNA fragmented by treatment with ultraviolet irradiation, hydroxylamine or cupferon (Reeves et al., FFMS Microbiol. Lett. 99, 193 - 198 (1992)). In this situation, killed protoplasts are referred to as donors, and viable protoplasts as acceptors.

Using dead donors cells can be advantageous in subsequently recognizing fused cells with hybrid genomes, as described below. Further, breaking up DNA in donor cells is advantageous for stimulating recombination with acceptor DNA. Optionally, acceptor and/or fused cells can also be briefly, but nonlethally, exposed to UV irradiation further to stimulate recombination.

Once formed, protoplasts can be stabilized in a variety of osmolytes and compounds such as sodium chloride, potassium chloride, sodium phosphate, potassium phosphate, sucrose, sorbitol in the presence of DTT. The combination of buffer, pH, reducing agent, and osmotic stabilizer can be optimized for different cell types. Protoplasts can be induced to fuse by treatment with a chemical such as PEG, calcium chloride or calcium propionate or electrofusion (Tsoneva, Acta Microbiologica Bulgaria 24, 53-59 (1989)). A method of cell fusion employing electric fields has also been described. See Chang US, 4,970,154. Conditions can be optimized for different strains.

The fused cells are heterokaryons containing genomes from two or more component protoplasts. Fused cells can be enriched from unfused parental cells by sucrose gradient sedimentation or cell sorting. The two nuclei in the heterokaryons can fuse (karyogamy) and homologous recombination can occur between the genomes. The



chromosomes can also segregate asymmetrically resulting in regenerated protoplasts that have lost or gained whole chromosomes. The frequency of recombination can be increased by treatment with ultraviolet irradiation or by use of strains overexpressing *recA* or other recombination genes, or the yeast *rad* genes, and cognate variants thereof in other species, or by the inhibition of gene products of *MutS*, *MutL*, or *MutD*. Overexpression can be either the result of introduction of exogenous recombination genes or the result of selecting strains, which as a result of natural variation or induced mutation, overexpress endogenous recombination genes. The fused protoplasts are propagated under conditions allowing regeneration of cell walls, recombination and segregation of recombinant genomes into progeny cells from the heterokaryon and expression of recombinant genes. This process can be reiteratively repeated to increase the diversity of any set of protoplasts or cells. After, or occasionally before or during, recovery of fused cells, the cells are screened or selected for evolution toward a desired property.

Thereafter a subsequent round of recombination can be performed by preparing protoplasts from the cells surviving selection/screening in a previous round. The protoplasts are fused, recombination occurs in fused protoplasts, and cells are regenerated from the fused protoplasts. This process can again be reiteratively repeated to increase the diversity of the starting population. Protoplasts, regenerated or regenerating cells are subject to further selection or screening.

Subsequent rounds of recombination can be performed on a split pool basis as described above. That is, a first subpopulation of cells surviving selection/screening from a previous round are used for protoplast formation. A second subpopulation of cells surviving selection/screening from a previous round are used as a source for DNA library preparation.

The DNA library from the second subpopulation of cells is then transformed into the protoplasts from the first subpopulation. The library undergoes recombination with the genomes of the protoplasts to form recombinant genomes. This process can be repeated several times in the absence of a selection event to increase the diversity of the cell

population. Cells are regenerated from protoplasts, and selection/screening is applied to regenerating or regenerated cells. In a further variation, a fresh library of nucleic acid fragments is introduced into protoplasts surviving selection/screening from a previous round.

Protoplast formation of donor and recipient strains, heterokaryon formation, karyogamy, recombination, and segregation of recombinant genomes into separate cells. Optionally, the recombinant genomes, if having a sexual cycle, can undergo further recombination with each other as a result of meiosis and mating. Recursive cycles of protoplast fusion, or recursive mating/meiosis is often used to increase the diversity of a cell population. After achieving a sufficiently diverse population via one of these forms of recombination, cells are screened or selected for a desired property. Cells surviving selection/screening can then be used as the starting materials in a further cycle of protoplasting or other recombination methods as noted herein.

Parasexual reproduction provides a further means for stochastic &/or non-stochastic mutagenesis genetic material between cells. This process allows recombination of parental DNA without involvement of mating types or gametes. Parasexual fusion occurs by hyphal fusion giving rise to a common cytoplasm containing different nuclei. The two nuclei can divide independently in the resulting heterokaryon but occasionally fuse. Fusion is followed by haploidization, which can involve loss of chromosomes and mitotic crossing over between homologous chromosomes. Protoplast fusion is a form of parasexual reproduction.

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8.8.3 SELECTION FOR HYBRID STRAINS

8.8.3.1 IDENTIFYING CELLS FORMED BY THE FUSION OF COMPONENTS OF PARENTAL CELLS FROM TWO OR MORE DISTINCT SUBPOPULATIONS

The invention provides selection strategies to identify cells formed by fusion of components from parental cells from two or more distinct subpopulations. Selection for hybrid cells is usually performed before selecting or screening for cells that have evolved (as a result of genetic exchange) to acquisition of a desired property. A basic premise of most such selection schemes is that two initial subpopulations have two distinct markers. Cells with hybrid genomes can thus be identified by selection for both markers.

8.8.3.2 METHOD WHERE ONE SUBPOPULATION HAS A MARKER

In one such scheme, at least one subpopulation of cells bears a selective marker attached to its cell membrane. Examples of suitable membrane markers include biotin, fluorescein and rhodamine. The markers can be linked to amide or thiol groups or through more specific derivatization chemistries, such as iodo-acetates, iodoacetamides, maleimides.

For example, a marker can be attached as follows. Cells or protoplasts are washed with a buffer (e.g., PBS), which does not interfere with the chemical coupling of a chemically active ligand which reacts with amino groups of lysines or N-terminal amino groups of membrane proteins. The ligand is either amine reactive itself (e.g., isothiocyanates, succinimidyl esters, sulfonyl chlorides) or is activated by a heterobifunctional linker (e.g. EMCS, SLAB, SPDP, SMB) to become amine reactive. The ligand is a molecule which is easily bound by protein derivatized magnetic beads or other capturing solid supports. For example, the ligand can be succinimidyl activated biotin (Molecular Probes Inc.: B-1606, B-2603, S-1515, S-1582). This linker is reacted with amino groups of proteins residing in and on the surface of a cell. The cells are then washed to remove excess labeling agent before contacting with cells from the second subpopulation bearing a second selective marker.

three subunits in three subpopulations), or more than one subunit can be expressed in the same subpopulation of cells (e.g., one subunit in one subpopulation, two subunits in a second subpopulation). In the case where more than two subunits are used, selection for the poolwise recombination of more than two protoplasts can be achieved.

Hybrid cells representing a combination of genomes of first, second or more subpopulation component cells can then be recognized by an assay for intact enzyme. Such an assay can be a binding assay, but is more typically a functional assay (e.g., capacity to metabolize a substrate of the enzyme). Enzymatic activity can be detected for example by processing of a substrate to a product with a fluorescent or otherwise easily detectable absorbance or emission spectrum. The individual subunits of a heteromultimeric enzyme used in such an assay preferably have no enzymic activity in dissociated form, or at least have significantly less activity in dissociated form than associated form. Preferably, the cells used for fusion lack an endogenous form of the heteromultimeric enzyme, or at least have significantly less endogenous activity than results from heteromultimeric enzyme formed by fusion of cells.

Penicillin acylase enzymes, cephalosporin acylase and penicillin acyltransferase are examples of suitable heteromultimeric enzymes. These enzymes are encoded by a single gene, which is translated as a proenzyme and cleaved by posttranslational autocatalytic proteolysis to remove a spacer endopeptide and generate two subunits, which associate to form the active heterodimeric enzyme. Neither subunit is active in the absence of the other subunit. However, activity can be reconstituted if these separated gene portions are expressed in the same cell by co-transformation. Other enzymes that can be used have subunits that are encoded by distinct genes (e.g., *faoA* and *faoB* genes encode 3-oxoacyl-CoA thiolase of *Pseudomonas fragi* (Biochem. J 328, 815-820 (1997))).

An exemplary enzyme is penicillin G acylase from *Escherichia coli*, which has two subunits encoded by a single gene. Fragments of the gene encoding the two subunits operably linked to appropriate expression regulation sequences are transfected into first and second subpopulations of cells, which lack endogenous penicillin acylase activity. A

cell formed by fusion of component cells from the first and second subpopulations expresses the two subunits, which assemble to form functional enzyme, e.g., penicillin acylase. Fused cells can then be selected on agar plates containing penicillin G, which is degraded by penicillin acylase.

In another variation, fused cells are identified by complementation. of auxotrophic mutants. Parental subpopulations of cells can be selected for known auxotrophic mutations. Alternatively, auxotrophic mutations in a starting population of cells can be generated spontaneously by exposure to a mutagenic agent. Cells with auxotrophic mutations are selected by replica plating on minimal and complete media. Lesions resulting in auxotrophy are expected to be scattered throughout the genome, in genes for amino acid, nucleotide, and vitamin biosynthetic pathways. After fusion of parental cells, cells resulting from fusion can be identified by their capacity to grow on minimal media. These cells can then be screened or selected for evolution toward a desired property. Further steps of mutagenesis generating fresh auxotrophic mutations can be incorporated in subsequent cycles of recombination and screening/selection.

In variations of the above method, de novo generation of auxotrophic mutations in each round of stochastic &/or non-stochastic mutagenesis can be avoided by reusing the same auxotrophs. For example, auxotrophs can be generated by transposon mutagenesis using a transposon bearing selective marker. Auxotrophs are identified by a screen such as replica plating. Auxotrophs are pooled, and a generalized transducing phage lysate is prepared by growth of phage on a population of auxotrophic cells. A separate population of auxotrophic cells is subjected to genetic exchange, and complementation is used to selected cells that have undergone genetic exchange and recombination. These cells are then screened or selected for acquisition of a desired property. Cells surviving screening or selection then have auxotrophic markers regenerated by introduction of the transducing transposon library. The newly generated auxotrophic cells can then be subject to further genetic exchange and screening/selection.

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In another variation, fused cells are identified by screening for a genomic marker present on one subpopulation of parental cells and an episomal marker present on a second subpopulation of cells. For example, a first subpopulation of yeast containing mitochondria can be used to complement a second subpopulation of yeast having a petite phenotype (i.e., lacking mitochondria).

8.8.4 LIPOSOME MEDIATED TRANSFERS

8.8.4.1.1 THE NUCLEIC ACIDS ARE ENCAPSULATED IN LIPOSOMES TO HELP UPTAKE BY PROTOPLASTS

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facilitate uptake by protoplasts. Liposome-mediated uptake of DNA by protoplasts is described in Redford et al., Mol. Gen. Genet. 184, 567-569 (1981). Liposomes can efficiently deliver large volumes of DNA to protoplasts (see Deshayes et al., FMOB J. 4, 2731-2737 (1985)).

See also, Philippot and Schuber (eds) (1995) *Liposomes as Tools in Basic Research and Industry* CRC press, Boca Raton, e.g., Chapter 9, Remy et al. "Gene Transfer with Cationic Amphiphiles." Further, the DNA can be delivered as linear fragments, which are often more recombinogenic than whole genomes. In some methods, fragments are mutated prior to encapsulation in liposomes. In some methods, fragments are combined with RecA and homologs, or nucleases (e.g., restriction endonucleases) before encapsulation in liposomes to promote recombination. Alternatively, protoplasts can be treated with lethal doses of nicking reagents and then fused. Cells which survive are those which are repaired by recombination with other genomic fragments, thereby providing a selection mechanism to select for recombinant (and therefore desirably diverse) protoplasts.

Filamentous fungi are particularly suited to performing the stochastic &/or non-stochastic mutagenesis methods described above. Filamentous fungi are divided into four main classifications based on their structures for sexual reproduction: Phycomycetes, Ascomycetes, Basidiomycetes and the Fungi Imperfecti. Phycomycetes (e.g., Rhizopus, Mucor) form sexual spores in sporangium.

Ascomycetes (e.g., *Aspergillus*, *Neurospora*, *Penicillium*) produce sexual spores in an ascus as a result of meiotic division. Asci typically contain 4 meiotic products, but some contain 8 as a result of additional mitotic division. Basidiomycetes include mushrooms, and smuts and form sexual spores on the surface of a basidium. In

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8.9.6 SPONTANEOUS FORMATION OF PROTOPLASTS

These fusants are used to generate mycelia and spores for the next round of stochastic &/or non-stochastic mutagenesis, and so forth. Eventually, progeny that spontaneously form protoplasts (i.e., without addition of cell wall degrading agent) are identified. As with other procedures noted herein, cells or protoplasts can be reiteratively fused and regenerated prior to performing any selection step to increase the diversity of

the resulting cells or protoplasts to be screened. Similarly, selected cells or protoplasts can be reiteratively fused and regenerated for one or several cycles without imposing selection on the resulting cellular or protoplast populations, thereby increasing the diversity of cells or protoplasts which are eventually screened. This process of performing multiple cycles of recombination interspersed with selection steps can be reiteratively repeated as desired.

8.9.7 ACQUISITION OR IMPROVEMENT OF GENES ENCODING IN BIOSYNTHETIC PATHWAYS; TRANSPORTER PROTEINS; AND METABOLIC FLUX

Another desired property is the acquisition and/or improvement of genes encoding enzymes in biosynthetic pathways, genes encoding transporter proteins, and genes encoding proteins involved in metabolic flux control. In this situation, genes of the pathway can be introduced into the fungus to be evolved either by genetic exchange with another strain of fungus possessing the pathway or by introduction of a fragment library from an organism possessing the pathway. Genetic material of these fungi can then be subjected to further stochastic &/or non-stochastic mutagenesis and screening/selection by the various procedures discussed in this application.

Shufflant strains of fungi are selected/screened for production of the compound produced by the metabolic pathway or precursors thereof.

8.9.8 INCREASED STABILITY TO EXTREME CONDITIONS

Another desired property is increasing the stability of fungi to extreme conditions such as heat. In this situation, genes conferring stability can be acquired by exchanging DNA with or transforming DNA from a strain that already has such properties.

Alternatively, the strain to be evolved can be subjected to random mutagenesis. Genetic material of the fungus to be evolved can be stochastic &/or non-stochastic mutagenized by any of the procedures described in this application, with shufflants being

The following table shows the results of the regression analysis for the dependent variable *ln* (*Y*) and the independent variables *X*₁, *X*₂, *X*₃, *X*₄, *X*₅, *X*₆, *X*₇, *X*₈, *X*₉, *X*₁₀, *X*₁₁, *X*₁₂, *X*₁₃, *X*₁₄, *X*₁₅, *X*₁₆, *X*₁₇, *X*₁₈, *X*₁₉, *X*₂₀, *X*₂₁, *X*₂₂, *X*₂₃, *X*₂₄, *X*₂₅, *X*₂₆, *X*₂₇, *X*₂₈, *X*₂₉, *X*₃₀, *X*₃₁, *X*₃₂, *X*₃₃, *X*₃₄, *X*₃₅, *X*₃₆, *X*₃₇, *X*₃₈, *X*₃₉, *X*₄₀, *X*₄₁, *X*₄₂, *X*₄₃, *X*₄₄, *X*₄₅, *X*₄₆, *X*₄₇, *X*₄₈, *X*₄₉, *X*₅₀, *X*₅₁, *X*₅₂, *X*₅₃, *X*₅₄, *X*₅₅, *X*₅₆, *X*₅₇, *X*₅₈, *X*₅₉, *X*₆₀, *X*₆₁, *X*₆₂, *X*₆₃, *X*₆₄, *X*₆₅, *X*₆₆, *X*₆₇, *X*₆₈, *X*₆₉, *X*₇₀, *X*₇₁, *X*₇₂, *X*₇₃, *X*₇₄, *X*₇₅, *X*₇₆, *X*₇₇, *X*₇₈, *X*₇₉, *X*₈₀, *X*₈₁, *X*₈₂, *X*₈₃, *X*₈₄, *X*₈₅, *X*₈₆, *X*₈₇, *X*₈₈, *X*₈₉, *X*₉₀, *X*₉₁, *X*₉₂, *X*₉₃, *X*₉₄, *X*₉₅, *X*₉₆, *X*₉₇, *X*₉₈, *X*₉₉, *X*₁₀₀, *X*₁₀₁, *X*₁₀₂, *X*₁₀₃, *X*₁₀₄, *X*₁₀₅, *X*₁₀₆, *X*₁₀₇, *X*₁₀₈, *X*₁₀₉, *X*₁₁₀, *X*₁₁₁, *X*₁₁₂, *X*₁₁₃, *X*₁₁₄, *X*₁₁₅, *X*₁₁₆, *X*₁₁₇, *X*₁₁₈, *X*₁₁₉, *X*₁₂₀, *X*₁₂₁, *X*₁₂₂, *X*₁₂₃, *X*₁₂₄, *X*₁₂₅, *X*₁₂₆, *X*₁₂₇, *X*₁₂₈, *X*₁₂₉, *X*₁₃₀, *X*₁₃₁, *X*₁₃₂, *X*₁₃₃, *X*₁₃₄, *X*₁₃₅, *X*₁₃₆, *X*₁₃₇, *X*₁₃₈, *X*₁₃₉, *X*₁₄₀, *X*₁₄₁, *X*₁₄₂, *X*₁₄₃, *X*₁₄₄, *X*₁₄₅, *X*₁₄₆, *X*₁₄₇, *X*₁₄₈, *X*₁₄₉, *X*₁₅₀, *X*₁₅₁, *X*₁₅₂, *X*₁₅₃, *X*₁₅₄, *X*₁₅₅, *X*₁₅₆, *X*₁₅₇, *X*₁₅₈, *X*₁₅₉, *X*₁₆₀, *X*₁₆₁, *X*₁₆₂, *X*₁₆₃, *X*₁₆₄, *X*₁₆₅, *X*₁₆₆, *X*₁₆₇, *X*₁₆₈, *X*₁₆₉, *X*₁₇₀, *X*₁₇₁, *X*₁₇₂, *X*₁₇₃, *X*₁₇₄, *X*₁₇₅, *X*₁₇₆, *X*₁₇₇, *X*₁₇₈, *X*₁₇₉, *X*₁₈₀, *X*₁₈₁, *X*₁₈₂, *X*₁₈₃, *X*₁₈₄, *X*₁₈₅, *X*₁₈₆, *X*₁₈₇, *X*₁₈₈, *X*₁₈₉, *X*₁₉₀, *X*₁₉₁, *X*₁₉₂, *X*₁₉₃, *X*₁₉₄, *X*₁₉₅, *X*₁₉₆, *X*₁₉₇, *X*₁₉₈, *X*₁₉₉, *X*₂₀₀, *X*₂₀₁, *X*₂₀₂, *X*₂₀₃, *X*₂₀₄, *X*₂₀₅, *X*₂₀₆, *X*₂₀₇, *X*₂₀₈, *X*₂₀₉, *X*₂₁₀, *X*₂₁₁, *X*₂₁₂, *X*₂₁₃, *X*₂₁₄, *X*₂₁₅, *X*₂₁₆, *X*₂₁₇, *X*₂₁₈, *X*₂₁₉, *X*₂₂₀, *X*₂₂₁, *X*₂₂₂, *X*₂₂₃, *X*₂₂₄, *X*₂₂₅, *X*₂₂₆, *X*₂₂₇, *X*₂₂₈, *X*₂₂₉, *X*₂₃₀, *X*₂₃₁, *X*₂₃₂, *X*₂₃₃, *X*₂₃₄, *X*₂₃₅, *X*₂₃₆, *X*₂₃₇, *X*₂₃₈, *X*₂₃₉, *X*₂₄₀, *X*₂₄₁, *X*₂₄₂, *X*₂₄₃, *X*₂₄₄, *X*₂₄₅, *X*₂₄₆, *X*₂₄₇, *X*₂₄₈, *X*₂₄₉, *X*₂₅₀, *X*₂₅₁, *X*₂₅₂, *X*₂₅₃, *X*₂₅₄, *X*₂₅₅, *X*₂₅₆, *X*₂₅₇, *X*₂₅₈, *X*₂₅₉, *X*₂₆₀, *X*₂₆₁, *X*₂₆₂, *X*₂₆₃, *X*₂₆₄, *X*₂₆₅, *X*₂₆₆, *X*₂₆₇, *X*₂₆₈, *X*₂₆₉, *X*₂₇₀, *X*₂₇₁, *X*₂₇₂, *X*₂₇₃, *X*₂₇₄, *X*₂₇₅, *X*₂₇₆, *X*₂₇₇, *X*₂₇₈, *X*₂₇₉, *X*₂₈₀, *X*₂₈₁, *X*₂₈₂, *X*₂₈₃, *X*₂₈₄, *X*₂₈₅, *X*₂₈₆, *X*₂₈₇, *X*₂₈₈, *X*₂₈₉, *X*₂₉₀, *X*₂₉₁, *X*₂₉₂, *X*₂₉₃, *X*₂₉₄, *X*₂₉₅, *X*₂₉₆, *X*₂₉₇, *X*₂₉

Another desired property is capacity of a fungus to grow under altered nutritional requirements (e.g., growth on particular carbon or nitrogen sources). Altering nutritional requirements is particularly valuable, e.g., for natural isolates of fungi that produce valuable commercial products but have esoteric and therefore expensive nutritional requirement. The strain to be evolved undergoes genetic exchange and/or transformation with DNA from a strain that has the desired nutritional requirements. The fungus to be evolved can then optionally be subjected to further stochastic &/or non-stochastic mutagenesis as described in this application and with recombinant strains being selected for capacity to grow in the desired nutritional circumstances. Optionally, the nutritional circumstances can be varied in successive rounds of stochastic &/or non-stochastic mutagenesis starting at close to the natural requirements of the fungus to be evolved and in subsequent rounds approaching the desired nutritional requirements.

Another desired property is acquisition of natural competence in a fungus. The procedure for acquisition of natural competence by stochastic &/or non-stochastic mutagenesis is generally described in PCT/US97/04494. The fungus to be evolved typically undergoes genetic exchange or transformation with DNA from a bacterial strain or fungal strain that already has this property.

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[Signature]

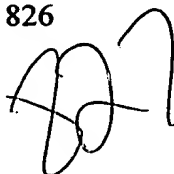
8.9.11 REDUCED OR INCREASED SECRETION OF PROTEASES AND DNASES

Another desired property is reduced or increased secretion of proteases and DNase. In this situation, the fungus to be evolved can acquire DNA by exchange or transformation from another strain known to have the desired property. Alternatively, the fungus to be evolved can be subject to random mutagenesis. The fungus to be evolved is stochastic &/or non-stochastic mutagenized as above. The presence of such enzymes, or lack thereof, can be assayed by contacting the culture media from individual isolates with a fluorescent molecule tethered to a support via a peptide or DNA linkage. Cleavage of the linkage releases detectable fluorescence to the media.

8.9.12 ALTERED TRANSPORTERS TO USE SECONDARY COMPONENTS

Another desired property is producing fungi with altered transporters (e. g., MDR). Such altered transporters are useful, for example, in fungi that have been evolved to produce new secondary metabolites, to allow entry of precursors required for synthesis of the new secondary metabolites into a cell, or to allow efflux of the secondary metabolite from the cell. Transporters can be evolved by introduction of a library of transporter variants into fungal cells and allowing the cells to recombine by sexual or parasexual recombination. To evolve a transporter with capacity to transport a precursor into the cells, cells are propagated in the presence of precursor, and cells are then screened for production of metabolite. To evolve a transporter with capacity to export a metabolite, cells are propagated under conditions supporting production of the metabolite, and screened for export of metabolite to culture medium.

A general method of fungal stochastic &/or non-stochastic mutagenesis is shown herein. Spores from a frozen stock, a lyophilized stock, or fresh from an agar plate are used to inoculate suitable liquid medium (1). Spores are germinated resulting in hyphal growth (2). Mycelia are harvested, and washed by filtration and/or centrifugation. Optionally the sample is pretreated with DTT to enhance protoplast formation (3). Protoplasting is performed in an osmotically stabilizing medium (e.g., 1 M NaCl/20mM MgSO₄, pH 5.8) by the addition of cell wall- degrading enzyme (e.g., Novozyme 234) (4).



mutations are temperature-sensitive. Temperature-sensitive strains can be propagated at the permissive temperature for purposes of selection and amplification and at a nonpermissive temperature for purposes of protoplast formation and fusion. A temperature sensitive strain *Neurospora crassa* os strain has been described which propagates as protoplasts when growth in osmotically stabilizing medium containing sorbose and polyoxin at nonpermissive temperature but generates whole cells on transfer to medium containing sorbitol at a permissive temperature. See US 4,873,196.

Other suitable strains can be produced by targeted mutagenesis of genes involved in chitin synthesis, glucan synthesis and other cell wall-related processes. Examples of such genes include CHT1, CHT2 and CAL1 (or CSD2) of *Saccharomyces cerevisiae* and *Candida* spp. (Georgopapadakou & Walsh 1996); ETGI/FKSI/CNDI/CWH53/PB RI and homologs in *S. cerevisiae*, *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, ChvAINDvA *Agrobacterium* and *Rhizobium*. Other examples are M4, orlB, orlC, MD, tsE, and bimG of *Aspergillus nidulans* (Borgia, J Bacteriol. 174, 3 77-3 89 (1992)).

Strains of *A. nidulans* containing OrlA1 or tse1 mutations lyse at restrictive temperatures. Lysis of these strains may be prevented by osmotic stabilization, and the mutations may be complemented by the addition of N-acetylglucosimine (GlcNac). BimG11 mutations are ts for a type 1 protein phosphatase (germlines of strains carrying this mutation lack chitin, and conidia swell and lyse). Other suitable genes are chsA, chsB, chsC, chsD and chsE of *Aspergillus fumigatus*; chs1 and chs2 of *Neurospora crassa*; *Phycomyces blakesleeanus* MM and chs 1, 2 and 3 of *S. cerevisiae*. Chs 1 is a non-essential repair enzyme; chs2 is involved in septum formation and chs3 is involved in cell wall maturation and bud ring formation.

Other useful strains include *S. cerevisiae* CLY (cell lysis) mutants such as ts strains (Paravicini et al., Mol. Cell Biol 12, 4896-4905 (1992)), and the CLY 15 strain which harbors a PKC 1 gene deletion. Other useful strains include strain VY 1160 containing a ts mutation in *srb* (encoding actin) (Schade et al. Acta Histochem. Suppl. 41, 193-200



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(1991)), and a strain with an *ses* mutation which results in increased sensitivity to cell-wall digesting enzymes isolated from snail gut (Metha & Gregory, *AppL Environ. Microbiol* 41, 992-999 (1981)). Useful strains of *C. albicans* include those with mutations in *chs1*, *chs2*, or *chs3* (encoding chitin synthetases), such as osmotic remedial conditional lethal mutants described by Payton & de Tiani, *Curr. Genet.* 17, 293-296 (1990); *C. utilis* mutants with increased sensitivity to cell-wall digesting enzymes isolated from snail gut (Metha & Gregory, 1981, *supra*); and *X. crassa* mutants *os-1*, *os-2*, *os-3*, *os-4*, *os-5*, and *os-6*. See, Selitrennikoff, *Antimicrob. Agents Chemother.* 23, 757-765 (1983). Such mutants grow and divide without a cell wall at 37 °C, but at 22 °C produce a cell wall.

Targeted mutagenesis can be achieved by transforming cells with a positive-negative selection vector containing homologous regions flanking a segment to be targeted, a positive selection marker between the homologous regions and a negative selection marker outside the homologous regions (see Capecchi, US 5,627,059). In a variation, the negative selection marker can be an antisense transcript of the positive selection marker (see US 5,527,674).

Other suitable cells can be selected by random mutagenesis or stochastic &/or non-stochastic mutagenesis procedures in combination with selection. For example, a first subpopulation of cells are mutagenized, allowed to recover from mutagenesis, subjected to incomplete degradation of cell walls and then contacted with protoplasts of a second subpopulation of cells. Hybrid cells bearing markers from both subpopulations are identified (as described above) and used as the starting materials in a subsequent round of stochastic &/or non-stochastic mutagenesis. This selection scheme selects both for cells with capacity for spontaneous protoplast formation and for cells with enhanced recombinogenicity.

In a further variation, cells having capacity for spontaneous protoplast formation can be crossed with cells having enhanced recombinogenicity evolved using other methods of the invention. The hybrid cells are particularly suitable hosts for whole genome stochastic &/or non-stochastic mutagenesis.

Cells with mutations in enzymes involved in cell wall synthesis or maintenance can undergo fusion simply as a result of propagating the cells in osmotic-protected culture due to spontaneous protoplast formation. If the mutation is conditional, cells are shifted to a nonpermissive condition. Protoplast formation and fusion can be accelerated by addition of promoting agents, such as PEG or an electric field (See Philipova & Venkov, Yeast 6, 205-212 (1990); Tsoneva et al., FFMS Microbiol Lett. 51, 61-65 (1989)).

8.10 PROCESS OF SEXUAL REPRODUCTION

Sexual reproduction provides a mechanism for stochastic &/or non-stochastic mutagenesis genetic material between cells. A sexual reproductive cycle is characterized by an alteration of a haploid phase and a diploid phase. Diploidy occurs when two haploid gamete nuclei fuse (karyogamy). The gamete nuclei can come from the same parental strains (self-fertile), such as in the homothallic fungi. In heterothallic fungi, the parental strains come from strains of different mating type.

A diploid cell converts to haploidy via meiosis, which essentially consists of two divisions of the nucleus accompanied by one division of the chromosomes. The products of one meiosis are a tetrad (4 haploid nuclei). In some cases, a mitotic division occurs after meiosis, giving rise to eight product cells. The arrangement of the resultant cells (usually enclosed in spores) resembles that of the parental strains. The length of the haploid and diploid stages differs in various fungi: for example, the Basidiomycetes and many of the Ascomycetes have a mostly haploid life cycle (that is, meiosis occurs immediately after karyogamy), whereas others (e.g., *Saccharomyces cerevisiae*) are diploid for most of their life cycle (karyogamy occurs soon after meiosis). Sexual reproduction can occur between cells in the same strain (selfing) or between cells from different strains (outcrossing).

Sexual dimorphism (dioecism) is the separate production of male and female organs on different mycelia. This is a rare phenomenon among the fungi, although a few examples are known. Heterothallism (one locus-two alleles) allows for outcrossing between crosscompatible strains which are self-incompatible. The simplest form is the

components of viruses, plasmids, YACS, HACs or BACs or can be introduced as they are, in which case all or most of the fragments lack an origin of replication. Use of viral fragments with single-stranded genomes offer the advantage of delivering fragments in single stranded form, which promotes recombination. The fragments can also be joined to a selective marker before introduction. Inclusion of fragments in a vector having an origin of replication affords a longer period of time after introduction into the cell in which fragments can undergo recombination with a cognate gene before being degraded or selected against and lost from the cell, thereby increasing the proportion of cells with recombinant genomes. Optionally, the vector is a suicide vector capable of a longer existence than an isolated DNA fragment but not capable of permanent retention in the cell line. Such a vector can transiently express a marker for a sufficient time to screen for or select a cell bearing the vector (e.g., because cells transduced by the vector are the target cell type to be screened in subsequent selection assays), but is then degraded or otherwise rendered incapable of expressing the marker. The use of such vectors can be advantageous in performing optional subsequent rounds of recombination to be discussed below. For example, some suicide vectors express a long-lived toxin which is neutralized by a short-lived molecule expressed from the same vector. Expression of the toxin alone will not allow vector to be established. Jense & Gerdes, Mol. Microbiol, 17, 205-210 (1995); Bernard et al., Gene 162, 159-160. Alternatively, a vector can be rendered suicidal by incorporation of a defective origin of replication (e.g. a temperature-sensitive origin of replication) or by omission of an origin of replication. Vectors can also be rendered suicidal by inclusion of negative selection markers, such as *ura3* in yeast or *sacB* in many bacteria.

These genes become toxic only in the presence of specific compounds. Such vectors can be selected to have a wide range of stabilities. A list of conditional replication defects for vectors which can be used, e.g., to render the vector replication defective is found, e.g., in Berg and Berg (1996), "Transposable element tools for microbial genetics" *Escherichia coli* and *Salmonella* Neidhardt. Washington, D.C., ASM Press. 2: 2588-2612. Similarly, a list of counter selectable markers, generally applicable to vector selection is also found in Berg and Berg, id. See also, LaRossa (1996), "Mutant selections linking



physiology, inhibitors, and genotypes" *Escherichia coli* and *Salmonella* F. C. Neidhardt. Washington, D.C., ASM Press. 2: 2527-2587.

After introduction into cells, the fragments can recombine with DNA present in the genome, or episomes of the cells by homologous, nonhomologous or site-specific recombination. For present purposes, homologous recombination makes the most significant contribution to evolution of the cells because this form of recombination amplifies the existing diversity between the DNA of the cells being transfected and the DNA fragments. For example, if a DNA fragment being transfected differs from a cognate or allelic gene at two positions, there are four possible recombination products, and each of these recombination products can be formed in different cells in the transformed population. Thus, homologous recombination of the fragment doubles the initial diversity in this gene. When many fragments recombine with corresponding cognate or allelic genes, the diversity of recombination products with respect to starting products increases exponentially with the number of mutations. Recombination results in modified cells having modified genomes and/or episomes. Recursive recombination prior to selection further increases diversity of resulting modified cells.

The variant cells, whether the result of natural variation, mutagenesis, or recombination are screened or selected to identify a subset of cells that have evolved toward acquisition of a new or improved property. The nature of the screen, of course, depends on the property and several examples will be discussed below. Typically, recombination is repeated before initial screening. Optionally, however, the screening can also be repeated before performing subsequent cycles of recombination. Stringency can be increased in repeated cycles of screening. The subpopulation of cells surviving screening are optionally subjected to a further round of recombination. In some instances, the further round of recombination is effected by propagating the cells under conditions allowing exchange of DNA between cells. For example, protoplasts can be formed from the cells, allowed to fuse, and regenerated. Cells with recombinant genomes are propagated from the fused protoplasts. Alternatively, exchange of DNA can be promoted by propagation of

[illegible]

In other methods, the further round of recombination is performed by a split and pool approach. That is, the surviving cells are divided into two pools. DNA is isolated from one pool, and if necessary amplified, and then transformed into the other pool.

8.11.2.1.3 SURVIVING CELLS ARE TRANSFECTED INTO FRESH DNA

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recombination with cognate genes in the surviving cells. If genes are introduced as components of a vector, compatibility of this vector with any vector used in a previous round of transfection should be considered. If the vector used in a previous round was a suicide vector, there is no problem of incompatibility. If, however, the vector used in a previous round was not a suicide vector, a vector having a different incompatibility origin should be used in the subsequent round. In all of these formats, further recombination generates additional diversity in the DNA component of the cells resulting in further modified cells.

The further modified cells are subjected to another round of screening/selection according to the same principles as the first round. Screening/selection identifies a subpopulation of further modified cells that have further evolved toward acquisition of the property. This subpopulation of cells can be subjected to further rounds of recombination and screening according to the same principles, optionally with the stringency of screening being increased at each round. Eventually, cells are identified that have acquired the desired property.

8.11.3 VARIATIONS

8.11.3.1 COATING WITH RecA TO ENRICH DIVERSITY OF HOMOLOGOUS RECOMBINATION

The frequency of homologous recombination between library fragments and cognate endogenous genes can be increased by coating the fragments with a recombinogenic protein before introduction into cells. See Pati et al., *Molecular Biology of Cancer* 1, 1 (1996); Sena & Zarling, *Nature Genetics* 3, 365 (1996); Revet et al., *J Mol. Biol.* 232, 779- 791 (1993); Kowalczkowski & Zarling in *Gene Targeting* (CRC 1995), Ch. 7. The recombinogenic protein promotes homologous pairing and/or strand exchange. The best characterized recA protein is from *E. coli* and is available from Pharmacia (Piscataway, NJ).



by conventional methods, such as chemical transformation or electroporation. In general, it can be desirable to coat the DNA with a RecA homolog isolated from the organism into which the coated DNA is being delivered. Recombination involves several cellular factors and the host RecA equivalent generally interacts better with other host factors than less closely related RecA molecules. The fragments undergo homologous recombination with cognate endogenous genes. Because of the increased frequency of recombination due to recombinase coating, the fragments need not be introduced as components of vectors. Fragments are sometimes coated with other nucleic acid binding proteins that promote recombination, protect nucleic acids from degradation, or target nucleic acids to the nucleus. Examples of such proteins includes *Agrobacterium* virE2 (Duffenberger et al., Proc. Natl. Acad. Sci. USA 86, 9154-9158 (1989)). Alternatively, the recipient strains are deficient in RecD activity. Single stranded ends can also be generated by 3'-5' exonuclease activity or restriction enzymes producing 5' overhangs.

8.11.3.2 AFFINITY CHROMATOGRAPHY WITH MutS TO ENRICH FOR FRAGMENTS HAVING AT LEAST ONE MISMATCH

The *E. coli* mismatch repair protein MutS can be used in affinity chromatography to enrich for fragments of double-stranded DNA containing at least one base of mismatch. The MutS protein recognizes the bubble formed by the individual strands about the point of the mismatch. See, e.g., Hsu & Chang, WO 9320233. The strategy of affinity enriching for partially mismatched duplexes can be incorporated into the present methods to increase the diversity between an incoming library of fragments and corresponding cognate or allelic genes in recipient cells.

MutS is used to increase diversity. The DNA substrates for enrichment are substantially similar to each other but differ at a few sites.

For example, the DNA substrates can represent complete or partial genomes (e.g., a chromosome library) from different individuals with the differences being due to polymorphisms. The substrates can also represent induced mutants of a wild type sequence.

The DNA substrates are pooled, restriction digested, and denatured to produce fragments of single-stranded DNA. The single-stranded DNA is then allowed to reanneal. Some single-stranded fragments reanneal with a perfectly matched complementary strand to generate perfectly matched duplexes. Other single-stranded fragments anneal to generate mismatched duplexes. The mismatched duplexes are enriched from perfectly matched duplexes by MutS chromatography (e.g., with MutS immobilized to beads). The mismatched duplexes recovered by chromatography are introduced into recipient cells for recombination with cognate endogenous genes as described above. MutS affinity chromatography increases the proportion of fragments differing from each other and the cognate endogenous gene. Thus, recombination between the incoming fragments and endogenous genes results in greater diversity.

A second strategy for MutS enrichment. In this strategy, the substrates for MutS enrichment represent variants of a relatively short segment, for example, a gene or cluster of genes, in which most of the different variants differ at no more than a single nucleotide. The goal of MutS enrichment is to produce substrates for recombination that contain more variations than sequences occurring in nature. This is achieved by fragmenting the substrates at random to produce overlapping fragments. The fragments are denatured and reannealed as in the first strategy. Reannealing generates some mismatched duplexes which can be separated from perfectly matched duplexes by MutS affinity chromatography. As before, MutS chromatography enriches for duplexes bearing at least a single mismatch. The mismatched duplexes are then stochastic &/or non-stochastic mutagenized into longer fragments. This is accomplished by cycles of denaturation, reannealing, and chain extension of partially annealed duplexes. After several such cycles, fragments of the same length as the original substrates are achieved, except that these fragments differ from each other at multiple sites. These fragments are then introduced into cells where they undergo recombination with cognate endogenous genes.

8.11.3.3 SUICIDE VECTOR ENRICHES MUTATIONS FOR CELLS THAT HAVE INTEGRATED THE VECTOR INTO THE HOST CHROMOSOME

The invention further provides methods of enriching for cells bearing modified genes relative to the starting cells. This can be achieved by introducing a DNA fragment library (e.g., a single specific segment or a whole or partial genomic library) in a suicide vector (i.e., lacking a functional replication origin in the recipient cell type) containing both positive and negative selection markers. Optionally, multiple fragment libraries from different sources (e.g., *B. subtilis*, *B. licheniformis* and *B. cereus*) can be cloned into different vectors bearing different selection markers. Suitable positive selection markers include neo^R , kanamycin^R , hyg , hisD , gpt , ble , tet^R . Suitable negative selection markers include hsv-tk , hpert , gpt , SacB ura3 and cytosine deaminase. A variety of examples of conditional replication vectors, mutations affecting vector replication, limited host range vectors, and counterselectable markers are found in Berg and Berg, *supra*, and LaRossa, *ibid.* and the references therein.

Another strategy for applying negative selection is to include a wild type *rpsL* gene (encoding ribosomal protein S12) in a vector for use in cells having a mutant *rpsL* gene conferring streptomycin resistance. The mutant form of *rpsL* is recessive in cells having wild type *rpsL*. Thus, selection for Sm resistance selects against cells having a wild type copy of *rpsL*. See Skorupski & Taylor, *Gene* 169, 47-52 (1996). Alternatively, vectors bearing only a positive selection marker can be used with one round of selection for cells expressing the marker, and a subsequent round of screening for cells that have lost the marker (e.g., screening for drug sensitivity). The screen for cells that have lost the positive selection marker is equivalent to screening against expression of a negative selection marker. For example, *Bacillus* can be transformed with a vector bearing a CAT gene and a sequence to be integrated. See Harwood & Cutting, *Molecular Biological Methods for*

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Bacillus, at pp. 31-33. Selection for chloramphenicol resistance isolates cells that have taken up vector. After a suitable period to allow recombination, selection for CAT sensitivity isolates cells which have lost the CAT gene. About 50% of such cells will have undergone recombination with the sequence to be integrated.

Suicide vectors bearing a positive selection marker and optionally, a negative selection marker and a DNA fragment can integrate into host chromosomal DNA by a single crossover at a site in chromosomal DNA homologous to the fragment. Recombination generates an integrated vector flanked by direct repeats of the homologous sequence. In some cells, subsequent recombination between the repeats results in excision of the vector and either acquisition of a desired mutation from the vector by the genome or restoration of the genome to wild type.

In the present methods, after transfer of the gene library cloned in a suitable vector, positive selection is applied for expression of the positive selection marker. Because nonintegrated copies of the suicide vector are rapidly eliminated from cells, this selection enriches for cells that have integrated the vector into the host chromosome. The cells surviving positive selection can then be propagated and subjected to negative selection, or screened for loss of the positive selection marker. Negative selection selects against cells expressing the negative selection marker. Thus, cells that have retained the integrated vector express the negative marker and are selectively eliminated. The cells surviving both rounds of selection are those that initially integrated and then eliminated the vector. These cells are enriched for cells having genes modified by homologous recombination with the vector. This process diversifies by a single exchange of genetic information. However, if the process is repeated either with the same vectors or with a library of fragments generated by PCR of pooled DNA from the enriched recombinant population, resulting in the diversity of targeted genes being enhanced exponentially each round of recombination. This process can be repeated recursively, with selection being performed as desired.

8.11.3.4 EXPLOITING KNOWN INFORMATION SUCH AS MAP LOCATION OR FUNCTION

In general, the above methods do not require knowledge of the number of genes to be optimized, their map location or their function. However, in some instances, where this information is available for one or more gene, it can be exploited. For example, if the property to be acquired by evolution is enhanced recombination of cells, one gene likely to be important is *recA*, even though many other genes, known and unknown, may make additional contributions. In this situation, the *recA* gene can be evolved, at least in part, separately from other candidate genes. The *recA* gene can be evolved by any of the methods of recursive recombination described in Section V. Briefly, this approach entails obtaining, diverse forms of a *recA* gene, allowing the forms to recombine, selecting recombinants having improved properties, and subjecting the recombinants to further cycles of recombination and selection. At any point in the individualized improvement of *recA*, the diverse forms of *recA* can be pooled with fragments encoding other genes in a library to be used in the general methods described herein. In this way, the library is seeded to contain a higher proportion of variants in a gene known to be important to the property sought to be acquired than would otherwise be the case.

In one example, a plasmid is constructed carrying a non-functional (mutated) version of a chromosomal gene such as *URA3*, where the wild-type gene confers sensitivity to a drug (in this case 5-fluoro orotic acid). The plasmid also carries a selectable marker (resistance to another drug such as kanamycin), and a library of *recA* variants. Transformation of the plasmid into the cell results in expression of the *recA* variants, some of which will catalyze homologous recombination at an increased rate. Those cells in which homologous recombination occurred are resistant to the selectable drug on the plasmid, and to 5-fluoro orotic acid because of the disruption of the chromosomal copy of this gene.

The *recA* variants which give the highest rates of homologous recombination are the most highly represented in a pool of homologous recombinants. The mutant *recA*

SECRET

In some stochastic &/or non-stochastic mutagenesis methods, DNA substrates are isolated from natural sources and are not easily manipulated by DNA modifying or polymerizing enzymes due to recalcitrant impurities, which poison enzymatic reactions. Such difficulties can be avoided by processing DNA substrates through a harvesting strain. The harvesting strain is typically a cell type with natural competence and a capacity for homologous recombination between sequences with substantial diversity (e.g., sequences exhibiting only 75% sequence identity). The harvesting strain bears a vector encoding a negative selection marker flanked by two segments respectively complementary to two segments flanking a gene or other region of interest in the DNA from a target organism. The harvesting strain is contacted with fragments of DNA from the target organism. Fragments are taken up by natural competence, or other methods described herein, and a fragment of interest from the target organism recombines with the vector of the harvesting strain causing loss of the negative selection marker. Selection against the negative marker allows isolation of cells that have taken up the fragment of interest.

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capable of homologous recombination of sequences showing only 75% sequence identity.

8.12 FURTHER APPLICATIONS

8.12.1 IMPROVED RECOMBINANCY

One goal of whole cell evolution is to generate cells having improved capacity for recombination. Such cells are useful for a variety of purposes in molecular genetics including the in vivo formats of recursive sequence recombination described in Section V. Almost thirty genes (e.g., *recA*, *recB*, *recC*, *recD*, *recE*, *recF*, *recG*, *recO*, *recQ*, *recR*, *recT*, *ruvA*, *ruvB*, *ruvC*, *sbcB*, *ssb*, *topA*, *gyrA* and *B*, *lig*, *polA*, *uvrD*, *E*, *recL*, *mutD*, *mutH*, *mutL*, *mutT*, *mutU*, *helD*) and DNA sites (e.g., *chi*, *recN*, *sbcC*) involved in genetic recombination have been identified in *E. coli*, and cognate forms of several of these genes have been found in other organisms (e.g., *rad51*, *rad55-rad57*, *Dmcl* in yeast (see Kowalczykowski et al., *Microbiol. Rev.* 58, 401-465 (1994); Kowalczykowski & Zarling, supra) and human homologs of *Rad51* and *Dmcl* have been identified (see Sandier et al., *Nucl. Acids Res.* 24, 2125-2132 (1996)). At least some of the *E. coli* genes, including *recA* are functional in mammalian cells, and can be targeted to the nucleus as a fusion with SV40 large T antigen nuclear targeting sequence (Reiss et al., *Proc. Mad. Acad. Sci. USA*, 93, 3094-3098 (1996)). Further, mutations in mismatch repair genes, such as *mutL*, *mutS*, *mutH* *mutT* relax homology requirements and allow recombination between more diverged sequences (Rayssiguier et al., *Nature* 342, 396-401 (1989)). The extent of recombination between divergent strains can be enhanced by impairing mismatch repair genes and stimulating SOS genes. Such can be achieved by use of appropriate mutant strains and/or growth under conditions of metabolic stress, which have been found to stimulate SOS and inhibit mismatch repair genes. Vulic et al., *Proc. Mad. Acad. Sci. USA* 94 (1997). In addition, this can be achieved by impairing the products of mismatch repair genes by exposure to selective inhibitors.

Starting substrates for recombination are selected according to the general principles described above. That is, the substrates can be whole genomes or fractions



thereof containing recombination genes or sites. Large libraries of essentially random fragments can be seeded with collections of fragments constituting variants of one or more known recombination genes, such as *recA*. Alternatively, libraries can be formed by mixing variant forms of the various known recombination genes and sites.

8.12.2 EXPRESSION OF *GFP* INDICATES CELL IS CAPABLE OF HOMOLOGOUS RECOMBINATION

The library of fragments is introduced into the recipient cells to be improved and recombination occurs, generating modified cells. The recipient cells preferably contain a marker gene whose expression has been disabled in a manner that can be corrected by recombination. For example, the cells can contain two copies of a marker gene bearing mutations at different sites, which copies can recombine to generate the wild type gene. A suitable marker gene is green fluorescent protein. A vector can be constructed encoding one copy of GFP having stop codons near the N-terminus, and another copy of GFP having stop codons near the C-terminus of the protein. The distance between the stop codons at the respective ends of the molecule is 500 bp and about 25% of recombination events result in active GFP. Expression of GFP in a cell signals that a cell is capable of homologous recombination to recombine in between the stop codons to generate a contiguous coding sequence. By screening for cells expressing GFP, one enriches for cells having the highest capacity for recombination. The same type of screen can be used following subsequent rounds of recombination. However, unless the selection marker used in previous round(s) was present on a suicide vector, subsequent round(s) should employ a second disabled screening marker within a second vector bearing a different origin of replication or a different positive selection marker to vectors used in the previous rounds.

8.12.3 INCREASED GENOME COPY NUMBER SO MORE CHROMOSOMES PER BACTERIAL CELL TO MAKE EVOLUTION QUICKER

The majority of bacterial cells in stationary phase cultures grown in rich media contain two, four or eight genomes. In minimal medium the cells contain one or two

genomes. The number of genomes per bacterial cell thus depends on the growth rate of the cell as it enters stationary phase. This is because rapidly growing cells contain multiple replication forks, resulting in several genomes in the cells after termination. The number of genomes is strain dependent, although all strains tested have more than one chromosome in stationary phase. The number of genomes in stationary phase cells decreases with time. This appears to be due to fragmentation and degradation of entire chromosomes, similar to apoptosis in mammalian cells. This fragmentation of genomes in cells containing multiple genome copies results in massive recombination and mutagenesis. Useful mutants may find ways to use energy sources that will allow them to continue growing. Multigenome or gene-redundant cells are much more resistant to mutagenesis and can be improved for a selected trait faster.

Some cell types, such as *Deinococcus radians* (Daly and Minton J Bacteriol 177, 5495-5505 (1995)) exhibit polyploidy throughout the cell cycle. This cell type is highly radiation resistant due to the presence of many copies of the genome. High frequency recombination between the genomes allows rapid removal of mutations induced by a variety of DNA damaging agents.

A goal of the present methods is to evolve other cell types to have increased genome copy number akin to that of *Deinococcus radians*. Preferably, the increased copy number is maintained through all or most of its cell cycle in all or most growth conditions. The presence of multiple genome copies in such cells results in a higher frequency of homologous recombination in these cells, both between copies of a gene in different genomes within the cell, and between a genome within the cell and a transfected fragment. The increased frequency of recombination allows the cells to be evolved more quickly to acquire other useful characteristics.

Starting substrates for recombination can be a diverse library of genes only a few of which are relevant to genomic copy number, a focused library formed from variants of gene(s) known or suspected to have a role in genomic copy number or a combination of the two. As a general rule one would expect increased copy number would be achieved by

8.12.4 EVOLVE SECRETION PATHWAYS FOR BETTER EFFICIENCY

8.12.5 EVOLVE TO MANUFACTURE DRUGS OR CHEMICALS

The protein (or metabolite) secretion pathways of bacterial and eukaryotic cells can be evolved to export desired molecules more efficiently, such as for the manufacturing of protein pharmaceuticals, small molecule drugs or specialty chemicals. Improvements in efficiency are particularly desirable for proteins requiring multisubunit assembly (such as antibodies) or extensive posttranslational modification before secretion.

The efficiency of secretion may depend on a number of genetic sequences including a signal peptide coding sequence, sequences encoding protein(s) that cleave or otherwise recognize the coding sequence, and the coding sequence of the protein being secreted. The latter may affect folding of the protein and the ease with which it can integrate into and traverse membranes. The bacterial secretion pathway in *E. coli* include the SecA, SecB, SecE, SecD and SecF genes. In *Bacillus subtilis*, the major genes are secA, secD, secE, secF, secY, ffh, ftsY together with five signal peptidase genes (sipS, sipT, sipU, sipV and sipW) (Kunst et al, supra). For proteins requiring posttranslational modification, evolution of genes effecting such modification may contribute to improved secretion. Likewise genes with expression products having a role in assembly of multisubunit proteins (e.g., chaperonins) may also contribute to improved secretion.

Selection of substrates for recombination follows the general principles discussed above. In this case, the focused libraries referred to above comprise variants of the known secretion genes. For evolution of prokaryotic cells to express eukaryotic proteins, the initial substrates for recombination are often obtained at least in part from eukaryotic sources.

Incoming fragments can undergo recombination both with chromosomal DNA in recipient cells and with the screening marker construct present in such cells (see below). The latter form of recombination is important for evolution of the signal coding sequence incorporated in the screening marker construct. Improved secretion can be screened by the inclusion of marker construct in the cells being evolved. The marker construct encodes a

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marker gene, operably linked to expression sequences, and usually operably linked to a signal peptide coding sequence. The marker gene is sometimes expressed as a fusion protein with a recombinant protein of interest. This approach is useful when one wants to evolve the recombinant protein coding sequence together with secretion genes.

8.12.6 EVOLVE SO PRODUCT IS TOXIC TO CELL UNLESS SECRETED

In one variation, the marker gene encodes a product that is toxic to the cell containing the construct unless the product is secreted. Suitable toxin proteins include diphtheria toxin and ricin toxin. Propagation of modified cells bearing such a construct selects for cells that have evolved to improve secretion of the toxin. Alternatively, the marker gene can encode a ligand to a known receptor, and cells bearing the ligand can be detected by FACS using labeled receptor. Optionally, such a ligand can be operably linked to a phospholipid anchoring sequence that binds the ligand to the cell membrane surface following secretion. In a further variation, secreted marker protein can be maintained in proximity with the cell secreting it by distributing individual cells into agar drops. This is done, e.g., by droplet formation of a cell suspension. Secreted protein is confined within the agar matrix and can be detected by e.g., FACS. In another variation, a protein of interest is expressed as a fusion protein together with beta- lactamase or alkaline phosphatase. These enzymes metabolize commercially available chromogenic substrates (e.g., X-gal), but do so only after secretion into the periplasm. Appearance of colored substrate in a colony of cells therefore indicates capacity to secrete the fusion protein and the intensity of color is related to the efficiency of secretion.

The cells identified by these screening and selection methods have the capacity to secrete increased amounts of protein. This capacity may be attributable to increased secretion and increased expression, or from increased secretion alone.

8.12.7 EVOLVE TO ACQUIRE INCREASED EXPRESSION OF RECOMBINANT PROTEIN

Expression Cells can also be evolved to acquire increased expression of a recombinant protein. The level of expression is, of course, highly dependent on the construct from which the recombinant protein is expressed and the regulatory sequences, such as the promoter, enhancer(s) and transcription termination site contained therein. Expression can also be affected by a large number of host genes having roles in transcription, posttranslational modification and translation. In addition, host genes involved in synthesis of ribonucleotide and amino acid monomers for transcription and translation may have indirect effects on efficiency of expression. Selection of substrates for recombination follows the general principles discussed above. In this case, focused libraries comprise variants of genes known to have roles in expression. For evolution of prokaryotic cells to express eukaryotic proteins, the initial substrates for recombination are often obtained, at least in part, from eukaryotic sources; that is eukaryotic genes encoding proteins such as chaperonins involved in secretion and/assembly of proteins. Incoming fragments can undergo recombination both with chromosomal DNA in recipient cells and with the screening marker construct present in such cells (see below).

Screening for improved expression can be effected by including a reporter construct in the cells being evolved. The reporter construct expresses (and usually secretes) a reporter protein, such as GFP, which is easily detected and nontoxic. The reporter protein can be expressed alone or together with a protein of interest as a fusion protein. If the reporter gene is secreted, the screening effectively selects for cells having either improved secretion or improved expression, or both.

8.12.8 EVOLVE PLANT CELLS TO ACQUIRE RESISTANCE

A further application of recursive sequence recombination is the evolution of plant cells, and transgenic plants derived from the same, to acquire resistance to pathogenic diseases (fungi, viruses and bacteria), insects, chemicals (such as salt, selenium, pollutants, pesticides, herbicides, or the like), including, e.g., atrazine or glyphosate, or to modify



chemical composition, yield or the like. The substrates for recombination can again be whole genomic libraries, fractions thereof or focused libraries containing variants of gene(s) known or suspected to confer resistance to one of the above agents. Frequently, library fragments are obtained from a different species to the plant being evolved.

The DNA fragments are introduced into plant tissues, cultured plant cells, plant microspores, or plant protoplasts by standard methods including electroporation (From et al., Proc. Natl. Acad. Sci. USA 82, 5824 (1985), infection by viral vectors such as cauliflower mosaic virus (CaMV) (Hohn et al., Molecular Biology of Plant Tumors, (Academic Press, New York, 1982) pp. 549-560; Howell, US 4,407,956), high velocity ballistic penetration by small particles with the nucleic acid either within the matrix of small beads or particles, or on the surface (Klein et al., Nature 327, 70-73 (1987)), use of pollen as vector (WO 85/01856), or use of *Agrobacterium tumefaciens* or *A. rhizogenes* carrying a T-DNA plasmid in which DNA fragments are cloned. The T-DNA plasmid is transmitted to plant cells upon infection by *Agrobacterium tumefaciens*, and a portion is stably integrated into the plant genome (Horsch et al., Science 233, 496-498 (1984); Fraley et al., Proc. Natl. Acad. Sci. USA 80, 4803 (1983)).

Diversity can also be generated by genetic exchange between plant protoplasts according to the same principles described below for fungal protoplasts. Procedures for formation and fusion of plant protoplasts are described by Takahashi et al., US 4,677,066; Akagi et al., US 5,360,725; Shimamoto et al., US 5,250,433; Cheney et al., US 5,426,040.

8.12.9 PLANT GENOME STOCHASTIC &/OR NON-STOCHASTIC MUTAGENESIS

Plant genome stochastic &/or non-stochastic mutagenesis allows recursive cycles to be used for the introduction and recombination of genes or pathways that confer improved properties to desired plant species. Any plant species, including weeds and wild cultivars, showing a desired trait, such as herbicide resistance, salt tolerance, pest resistance, or temperature tolerance, can be used as the source of DNA that is introduced into the crop or horticultural host plant species.



Genomic DNA prepared from the source plant is fragmented (e.g. by DNaseI, restriction enzymes, or mechanically) and cloned into a vector suitable for making plant genomic libraries, such as pGA482 (An. G., 1995, Methods Mol. Biol. 44:47-58). This vector contains the *A. tumefaciens* left and right borders needed for gene transfer to plant cells and antibiotic markers for selection in *E. coli*, *Agrobacterium*, and plant cells. A multicloning site is provided for insertion of the genomic fragments. A cos sequence is present for the efficient packaging of DNA into bacteriophage lambda heads for transfection of the primary library into *E. coli*. The vector accepts DNA fragments of 25-40 kb.

The primary library can also be directly electroporated into an *A. tumefaciens* or *A. rhizogenes* strain that is used to infect and transform host plant cells (Main, GD et al., 1995, Methods Mol. Biol. 44:405-412). Alternatively, DNA can be introduced by electroporation or PEG-mediated uptake into protoplasts of the recipient plant species (Bilang et al. (1994) Plant Mol. Biol Manual, Kluwer Academic Publishers, A1: 1- 16) or by particle bombardment of cells or tissues (Christou, *ibid*, A2:1-15). If necessary, antibiotic markers in the T-DNA region can be eliminated, as long as selection for the trait is possible, so that the final plant products contain no antibiotic genes.

Stably transformed whole cells acquiring the trait are selected on solid or liquid media containing the agent to which the introduced DNA confers resistance or tolerance. If the trait in question cannot be selected for directly, transformed cells can be selected with antibiotics and allowed to form callus or regenerated to whole plants and then screened for the desired property.

The second and further cycles consist of isolating genomic DNA from each transgenic line and introducing it into one or more of the other transgenic lines. In each round, transformed cells are selected or screened for incremental improvement. To speed the process of using multiple cycles of transformation, plant regeneration can be deferred until the last round. Callus tissue generated from the protoplasts or transformed tissues can



as, e.g., rice (Chen, CC 1977 In Vitro. 13: 484-489), tobacco (Atanassov, I. et al. 1998 Plant Mol Biol. 38:1169-1178), Tradescantia (Savage JRK and Papworth DG. 1998 Mutat Res. 422:313-322), Arabidopsis (Park SK et al. 1998 Development. 125:3789- 3799), sugar beet (Majewska-Sawka A and Rodrigues-Garcia NE 1996 J Cell Sci. 109:859-866), Barley (Olsen FL 1991 Hereditas 115:255-266) and oilseed rape (Boutillier KA et al. 1994 Plant Mol Biol. 26:1711-1723).

The plants derived from microspores are predominantly haploid or diploid (infrequently polyploid and aneuploid). The diploid plants are homozygous and fertile and can be generated in a relatively short time. Microspores obtained from F1 hybrid plants represent great diversity, thus being an excellent model for studying recombination. In addition, microspores can be transformed with T-DNA introduced by agrobacterium or other available means and then regenerated into individual plants. Furthermore, protoplasts can be made from microspores and they can be fused similar to what occur in fungi and bacteria.

Microspores, due to their complex ploidy and regenerating ability, provide a tool for plant whole genome stochastic &/or non-stochastic mutagenesis. For example, if pollens from 4 parents are collected 4 and pooled, and then used to randomly pollinate the parents, the progenies should have $2^4 = 16$ possible combinations. Assuming this plant has 7 chromosomes, microspores collected from the 16 progenies will represent $2^7 \times 16 = 2048$ possible chromosomal combinations. This number is even greater if meiotic processes occur. When diploid, homozygous embryos are generated from these microspores, in many cases, they are screened for desired phenotypes, such as herbicide- or disease- resistant. In addition, for plant oil composition these embryos can be dissected into two halves: one for analysis the other for regeneration into a viable plant. Protoplasts generated from microspores (especially the haploid ones) are pooled and fused. Microspores obtained from plants generated by protoplast fusion are pooled and fused again, increasing the genetic diversity of the resulting microspores. Microspores can be subjected to mutagenesis in various ways, such as by chemical mutagenesis, radiation-induced mutagenesis and, e.g., t-DNA transformation, prior to fusion or regeneration. New

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by recursive sequence recombination. Suitable substrates for recombination include regulatory sequences such as promoters and enhancers from milk-protein genes from different species or individual animals. Cycles of recombination can be performed in vitro or in vivo by any of the formats discussed. Screening is performed in vivo on cultures of mammary-gland derived cells, such as HC11 or MacT, transfected with transgenes and reporter constructs such as those discussed above. After several cycles of recombination and screening, transgenes resulting in the highest levels of expression and secretion are extracted from the mammary gland tissue culture cells and used to transfect embryonic cells, such as zygotes and embryonic stem cells, which are matured into transgenic animals.

8.13.2 OPTIMIZE WHOLE ANIMAL BY TRANSFORMING INTO EMBRYONIC CELLS GENE OF DESIRED TRAIT

8.13.2.1 GROWTH HORMONE

In this approach, libraries of incoming fragments are transformed into embryonic cells, such as ES cells or zygotes. The fragments can be variants of a gene known to confer a desired property, such as growth hormone. Alternatively, the fragments can be partial or complete genomic libraries including many genes. Fragments are usually introduced into zygotes by microinjection as described by Gordon et al., *Methods Enzymol.* 10 1, 414 (1984); Hogan et al., *Manipulation of the Mouse Embryo: A Laboratory Manual* (C.S.H.L. N.Y., 1986) (mouse embryo),- and Hammer et al., *Nature* 315, 680 (1985) (rabbit and porcine embryos); Gandolfi et al., *J Reprod. Fert.* 81, 23-28 (1987); Rexroad et al., *J Anim. Sci.* 66, 947-953 (1988) (ovine embryos) and Eyestone et al., *J Reprod. Fert.* 85, 715-720 (1989); Camous et al., *J Reprod. Fert.* 72, 779- 785 (1984); and Heyman et al., *Theriogenology* 27, 5968 (1987) (bovine embryos). Zygotes are then matured and introduced into recipient female animals which gestate the embryo and give birth to a transgenic offspring.

Alternatively, transgenes can be introduced into embryonic stem cells (ES).

These cells are obtained from preimplantation embryos cultured in vitro. Bradley et al., *Nature* 309, 255-258 (1984). Transgenes can be introduced into such cells by

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electroporation or microinjection. Transformed ES cells are combined with blastocysts from a non-human animal. The ES cells colonize the embryo and in some embryos form the germ line of the resulting chimeric animal. See Jaenisch, Science, 240, 1468-1474 (1988).

Regardless whether zygotes or ES are used, screening is performed on whole animals for a desired property, such as increased size and/or growth rate. DNA is extracted from animals having evolved toward acquisition of the desired property. This DNA is then used to transfect further embryonic cells. These cells can also be obtained from animals that have acquired toward the desired property in a split and pool approach. That is, DNA from one subset of such animals is transformed into embryonic cells prepared from another subset of the animals. Alternatively, the DNA from animals that have evolved toward acquisition of the desired property can be transfected into fresh embryonic cells. In either alternative, transfected cells are matured into transgenic animals, and the animals subjected to a further round of screening for the desired property.

Initially, a library is prepared of variants of a growth hormone gene. The variants can be natural or induced. The library is coated with recA protein and transfected into fertilized fish eggs. The fish eggs then mature into fish of different sizes. The growth hormone gene fragment of genomic DNA from large fish is then amplified by PCR and used in the next round of recombination. Alternatively, fish γ -IFN is evolved to enhance resistance to viral infections as described below.

8.13.2.2 EVOLUTION OF IMPROVED HORMONES FOR EXPRESSION IN TRANSGENIC ANIMALS

8.13.3 TO CREATE ANIMALS WITH IMPROVED TRAITS

Evolution of improved hormones for expression in transgenic animals (e.g., Fish) to create animals with improved traits. Hormones and cytokines are key regulators of size, body weight, viral resistance and many other commercially important traits. DNA stochastic &/or non-stochastic mutagenesis is used to rapidly evolve the genes for these

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proteins using in vitro assays. This was demonstrated with the evolution of the human alpha interferon genes to have potent antiviral activity on murine cells. Large improvements in activity were achieved in two cycles of family stochastic &/or non-stochastic mutagenesis of the human IFN genes.

In general, a method of increasing resistance to virus infection in cells can be performed by first introducing a stochastic &/or non-stochastic mutagenized library comprising at least one stochastic &/or non-stochastic mutagenized interferon gene into animal cells to create an initial library of animal cells or animals. The initial library is then challenged with the virus. Animal cells or animals are selected from the initial library which are resistant to the virus and a plurality of transgenes from a plurality of animal cells or animals which are resistant to the virus are recovered. The plurality of transgenes is recovered to produce an evolved library of animal cells or animals which is again challenged with the virus. Cells or animals are selected from the evolved library the which are resistant to the virus.

For example, genes evolved with in vitro assays are introduced into the germplasm of animals or plants to create improved strains. One limitation of this procedure is that in vitro assays are often only crude predictors of in vivo activity. However, with improving methods for the production of transgenic plants and animals, one can now marry whole organism breeding with molecular breeding. The approach is to introduce stochastic &/or non-stochastic mutagenized libraries of hormone genes into the species of interest. This can be done with a single gene per transgenic or with pools of genes per transgenic. Progeny are then screened for the phenotype of interest. In this case, stochastic &/or non-stochastic mutagenized libraries of interferon genes (alpha IFN for example) are introduced into transgenic fish. The library of transgenic fish are challenged with a virus. The most resistant fish are identified (i.e. either survivors of a lethal challenge; or those that are deemed most 'healthy' after the challenge). The IFN transgenes are recovered by PCR and stochastic &/or non-stochastic mutagenized in either a poolwise or a pairwise fashion. This generates an evolved library of IFN genes. A second library of transgenic fish is created and the process is repeated. In this way, IFN is evolved for improved

1. The first group of people who are not allowed to enter the country are those who are on the "no-fly" list. This list is maintained by the Department of Homeland Security and includes individuals who are considered a threat to national security.

In one embodiment, BHK-21 (A fibroblast cell line from hamster) can be transfected with the stochastic &/or non-stochastic mutagenized WN-expression plasmids. Active recombinant IFN is produced and then purified by WGA agarose affinity chromatography (Tamai, et al. 1993 Biochim Ciophys Acta. supra). The antiviral activity of IFN can be measured on fish cells challenged by rhabdovirus. Tami et al. (1993) "Purification and characterization of interferon-like antiviral protein derived from flatfish (*Paralichthys olivaceus*) lymphocytes immortalized by oncogenes. " Cytotechnology 1993; 11 (2):121-131).

POOLWISE RECURSIVE BREEDING The present invention provides a procedure for generating large combinatorial libraries of higher eukaryotes, plants, fish, domesticated animals, etc. In addition to the procedures outlined above, poolwise

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The process is repeated with the subsequent progeny for several generations, with the final progeny being a combinatorial organism library with each member having genetic information originating from many if not all of the starting "parents." This process generates large diverse organism libraries on which many selections and or screens can be imparted, and it does not require sophisticated in vitro manipulation of genes. However, it results in the creation of useful new strains (perhaps well diluted in the population) in a much shorter time frame than such organisms could be generated using a classical targeted breeding approach.

These libraries are generated relatively quickly (e.g., typically in less than three years for most plants of commercial interest, with six cycles or less of recursive breeding being sufficient to generate desired diversity). An additional benefit of these methods is that the resulting libraries provide organismal diversity in areas, such as agriculture, aquaculture, and animal husbandry, that are currently genetically homogeneous.

Examples of these methods for several organisms are described below.

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8.13.5 PLANTS

Plants A population of plants, for example all of the different corn strains in a commercial seed/germplasm collection, are grown and the pollen from the entire population is harvested and pooled. This mixed pollen population is then used to "self" fertilize the same population. Self pollination is prevented, so that the fertilization is combinatorial. The cross results in all pairwise crosses possible within the population, and the resulting seeds result in many of the possible outcomes of each of these pairwise crosses. The seeds from the fertilized plants are then harvested, pooled, planted, and the pollen is again harvested, pooled, and used to "self" fertilize the population. After only several generations, the resulting population is a very diverse combinatorial library of corn. The seeds from this library are harvested and screened for desirable traits, e.g., salt tolerance, growth rate, productivity, yield, disease resistance, etc. Essentially any plant collection can be modified by this approach. Important commercial crops include both monocots and dicots. Monocots include plants in the grass family (Gramineae), such as plants in the sub families Fetucoideae and Poacoideae, which together include several hundred genera including plants in the genera Agrostis, Phleum, Dactylis, Sorghum, Setaria, Zea (e.g., corn), Oryza (e.g., rice), Triticum (e.g., wheat), Secale (e.g., rye), Avena (e.g., oats), Hordeum (e.g., barley), Saccharum, Poa, Festuca, Stenotaphrum, Cynodon, Coix, the Olyreae, Phareae and many others. Plants in the family Gramineae are a particularly preferred target plants for the methods of the invention.

Additional preferred targets include other commercially important crops, e.g., from the families Compositae (the largest family of vascular plants, including at least 1,000 genera, including important commercial crops such as sunflower), and Leguminosae or "pea family," which includes several hundred genera, including many commercially valuable crops such as pea, beans, lentil, peanut, yam bean, cowpeas, velvet beans, soybean, clover, alfalfa, lupine, vetch, lotus, sweet clover, wisteria, and sweetpea. Common crops applicable to the methods of the invention include Zea mays, rice, soybean, sorghum, wheat, oats, barley, millet, sunflower, and canola.

This process can also be carried out using pollen from different species or more divergent strains (e.g., crossing the ancient grasses with corn). Different plant species can

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be forced to cross. Only a few plants from an initial cross would have to result in order to make the process viable. These few progeny, e.g., from a cross between soy bean and corn, would generate pollen and eggs, each of which would represent a different meiotic outcome from the recombination of the two genomes. The pollen would be harvested and used to "self pollinate the original progeny. This process would then be carried out recursively. This would generate a large family stochastic &/or non-stochastic mutagenized library of two or more species, which could be subsequently screened.

8.13.6 FISH

Fish The natural tendency of fish to lay their eggs outside of the body and to have a male cover those eggs with sperm provides another opportunity for a split pooled breeding strategy. The eggs from many different fish, e.g., salmon from different fisheries about the world, can be harvested, pooled, and then fertilized with similarly collected and pooled salmon sperm. The fertilization will result in all of the possible pairwise matings of the starting population. The resulting progeny is then grown and again the sperm and eggs are harvested, and pooled, with each egg and sperm representing a different meiotic outcome of the different crosses. The pooled sperm are then used to fertilize the pooled eggs and the process is carried out recursively. After several generations the resulting progeny can then be subjected to selections and screens for desired properties, such as size, disease resistance, etc.

8.13.7 ANIMALS

Animals The advent of in vitro fertilization and surrogate motherhood provides a means of whole genome stochastic &/or non-stochastic mutagenesis in animals such as mammals. As with fish, the eggs and the sperm from a population, for example from all slaughter cows, are collected and pooled. The pooled eggs are then in vitro fertilized with the pooled sperm. The resulting embryos are then returned to surrogate mothers for development. As above, this process is repeated recursively until a large diverse population is generated that can be screened for desirable traits.

A technically feasible approach would be similar to that used for plants. In this

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Recursive sequence recombination can be used to simulate natural evolution of pathogenic microorganisms in response to exposure to a drug under test. Using recursive sequence recombination, evolution proceeds at a faster rate than in natural evolution. One measure of the rate of evolution is the number of cycles of recombination and screening required until the microorganism acquires a defined level of resistance to the drug. The information from this analysis is of value in comparing the relative merits of different drugs and in particular, in predicting their long term efficacy on repeated administration.

The pathogenic microorganisms used in this analysis include the bacteria that are a common source of human infections, such as chlamydia, rickettsial bacteria, mycobacteria, staphylococci, streptococci, pneumococci, meningococci and gonococci, klebsiella, proteus, serratia, pseudomonas, legionella, diphtheria, salmonella, bacilli, cholera, tetanus, botulism, anthrax, plague, leptospirosis, and Lyme disease bacteria.

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to the drug. After transformation and propagation of bacteria for an appropriate period to allow for recombination to occur and recombinant genes to be expressed, the bacteria are screened by exposing them to the drug under test and then collecting survivors. Surviving bacteria are subject to further rounds of recombination. The subsequent round can be effected by a split and pool approach in which DNA from one subset of surviving bacteria is introduced into a second subset of bacteria. Alternatively, a fresh library of DNA fragments can be introduced into surviving bacteria. Subsequent round(s) of selection can be performed at increasing concentrations of drug, thereby increasing the stringency of selection.

8.14.1 BIOSYNTHESIS

Metabolic engineering can be used to alter organisms to optimize the production of practically any metabolic intermediate, including antibiotics, vitamins, amino acids such as phenylalanine and aromatic amino acids, ethanol, butanol, polymers such as xanthan gum and bacterial cellulose, peptides, and lipids. When such compounds are already produced by a host, the recursive sequence recombination techniques described above can be used to optimize production of the desired metabolic intermediate, including such features as increasing enzyme substrate specificity and turnover number, altering metabolic fluxes to reduce the concentrations of toxic substrates or intermediates, increasing resistance of the host to such toxic compounds, eliminating, reducing or altering the need for inducers of gene expression/activity, increasing the production of enzymes necessary for metabolism, etc.

Enzymes can also be evolved for improved activity in solvents other than water. This is useful because intermediates in chemical syntheses are often protected by blocking groups which dramatically affect the solubility of the compound in aqueous solvents. Many compounds can be produced by a combination of pure chemical and enzymically catalyzed reactions. Performing enzymic reactions on almost insoluble substrates is clearly very inefficient, so the availability of enzymes that are active in other solvents will be of great use. One example of such a scheme is the evolution of a para- nitrobenzyl esterase to remove protecting groups from an intermediate in loracarbef synthesis (Moore, J.C. and

Arnold, F.H. *Nature Biotechnology* 14:458-467 (1996)). In this case alternating rounds of error-prone PCR and colony screening for production of a fluorescent reporter from a substrate analogue were used to generate a mutant esterase that was 16-fold more active than the parent molecule in 30% dimethylformamide. No individual mutation was found to contribute more than a 2-fold increase in activity, but it was the combination of a number of mutations which led to the overall increase.

Structural analysis of the mutant protein showed that the amino acid changes were distributed throughout the length of the protein in a manner that could not have been rationally predicted. Sequential rounds of error-prone PCR have the problem that after each round all but one mutant is discarded, with a concomitant loss of information contained in all the other beneficial mutations. Recursive sequence recombination avoids this problem, and would thus be ideally suited to evolving enzymes for catalysis in other solvents, as well as in conditions where salt concentrations or pH were different from the original enzyme optima.

In addition, the yield of almost any metabolic pathway can be increased, whether consisting entirely of genes endogenous to the host organisms or all or partly heterologous genes. Optimization of the expression levels of the enzymes in a pathway is more complex than simply maximizing expression. In some cases regulation, rather than constitutive expression of an enzyme may be advantageous for cell growth and therefore for product yield, as seen for production of phenylalanine (Backman et al. *Ann. NY Acad. Sci.* 589:16-24 (1990)) and 2-keto-L- gluconic acid (Anderson et al. U.S. 5,032,514). In addition, it is often advantageous for industrial purposes to express proteins in organisms other than their original hosts. New host strains may be preferable for a variety of reasons, including ease of cloning and transformation, pathogenicity, ability to survive in particular environments and a knowledge of the physiology and genetics of the organisms. However, proteins expressed in heterologous organisms often show markedly reduced activity for a variety of reasons including inability to fold properly in the new host (Sarthý et al. *Appl. Environ. Micro.* 53:1996-2000 (1987)). Such difficulties can indeed be overcome by the recursive sequence recombination strategies of the instant invention.

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First, antibiotic synthesis enzymes can be "evolved" together with transport systems that allow entry of compounds used as antibiotic precursors to improve uptake and incorporation of function-altering artificial side chain precursors. For example, penicillin V is produced by feeding *Penicillium* the artificial side chain precursor phenoxyacetic acid, and LY146032 by feeding *Streptomyces roseosporus* decanoic acid (Hopwood, Phil. Trans. R. Soc. Lond. B 324:549-562 (1989)). Poor precursor uptake and poor incorporation by the synthesizing enzyme often lead to inefficient formation of the desired product. Recursive sequence recombination of these two systems can increase the yield of desired product.

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Second, the expression of heterologous genes transferred from one antibiotic synthesizing organism to another can be optimized. The newly introduced enzyme(s) act on secondary metabolites in the host cell, transforming them into new compounds with novel properties. Using traditional methods, introduction of foreign genes into antibiotic synthesizing hosts has already resulted in the production of novel hybrid antibiotics. Examples include mederrhodin, dihydrogranatirhodin, 6-deoxyerythromycin A, isovalerylspiramycin and other hybrid macrolides (Cameron et. al. Appl. Biochem. Biotechnol. 38:105-140 (1993)). The recursive sequence recombination techniques of the instant invention can be used to optimize expression of the foreign genes, to stabilize the enzyme in the new host cell, and to increase the activity of the introduced enzyme against its new substrates in the new host cell. In some embodiments of the invention, the host genome may also be so optimized.

Third, the substrate specificity of an enzyme involved in secondary metabolism can be altered so that it will act on and modify a new compound or so that its so activity is changed and it acts at a different subset of positions of its normal substrate. Recursive sequence recombination can be used to alter the substrate specificities of enzymes. Furthermore, in addition to recursive sequence recombination of individual enzymes being a strategy to generate novel antibiotics, recursive sequence recombination of entire pathways, by altering enzyme ratios, will alter metabolite fluxes and may result, not only in increased antibiotic synthesis, but also in the synthesis of different antibiotics. This can be deduced from the observation that expression of different genes from the same cluster in a foreign host leads to different products being formed (see p. 80 in Hutchinson et. al., (1991) Ann NY Acad Sci, 646:78-93).

Recursive sequence recombination of the introduced gene clusters may result in a variety of expression levels of different proteins within the cluster (because it produces different combinations of, in this case regulatory, mutations). This in turn may lead to a variety of different end products. Thus, "evolution" of an existing antibiotic synthesizing pathway could be used to generate novel antibiotics either by modifying the rates or substrate specificities of enzymes in that pathway.


macrolide antibiotics, reviewed in Cameron et al. *Appl. Biochem. Biotechnol.* 38:105-140 (1993).

8.14.3 BIOSYNTHESIS TO REPLACE CHEMICAL SYNTHESIS OF ANTIBIOTICS

In yet another example, penicillin amidase expressed in *E. coli* is a key enzyme in the production of penicillin G derivatives. The enzyme is generated from a precursor peptide and tends to accumulate as insoluble aggregates in the periplasm unless non-metabolizable sugars are present in the medium (Scherrer et al. , Appl. Microbiol. Biotechnol. 42:85-91 (1994)). Evolution of this enzyme through the methods of the instant invention could be used to generate an enzyme that folds better, leading to a higher level of active enzyme expression.

In yet another example, Penicillin G acylase covalently linked to agarose is used in the synthesis of penicillin G derivatives. The enzyme can be stabilized for increased activity, longevity and/or thermal stability by chemical modification (Fernandez-Lafuente et. al. *Enzyme Microb. Technol.* 14:489-495 (1992)). Increased thermal stability is an

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especially attractive application of the recursive sequence recombination techniques of the instant invention, which can obviate the need for the chemical modification of such enzymes. Selection for thermostability can be performed in vivo in *E. coli* or in thermophiles at higher temperatures. In general, thermostability is a good first step in enhancing general stabilization of enzymes. Random mutagenesis and selection can also be used to adapt enzymes to function in non-aqueous solvents (Arnold Curr Opin Biotechnol, 4:450-455 (1993); Chen et. al. Proc. Natl. Acad. Sci. U.S.A., 90:5618-5622 (1993)). Recursive sequence recombination represents a more powerful (since recombinogenic) method of generating mutant enzymes that are stable and active in non-aqueous environments. Additional screening can be done on the basis of enzyme stability in solvents.

8.14.4 POLYKETIDES

Polyketides include antibiotics such as tetracycline and erythromycin, anti-cancer agents such as daunomycin, immunosuppressants such as FK506 and rapamycin and veterinary products such as monesin and avermectin. Polyketide synthases (PKS's) are multifunctional enzymes that control the chain length, choice of chain-building units and reductive cycle that generates the huge variation in naturally occurring polyketides. Polyketides are built up by sequential transfers of "extender units" (fatty acyl CoA groups) onto the appropriate starter unit (examples are acetate, coumarate, propionate and malonamide). The PKS's determine the number of condensation reactions and the type of extender groups added and may also fold and cyclize the polyketide precursor. PKS's reduce specific β -keto groups and may dehydrate the resultant β -hydroxyls to form double bonds. Modifications of the nature or number of building blocks used, positions at which β -keto groups are reduced, the extent of reduction and different positions of possible cyclizations, result in formation of different final products. Polyketide research is currently focused on modification and inhibitor studies, site directed mutagenesis and 3-D structure elucidation to lay the groundwork for rational changes in enzymes that will lead to new polyketide products.

Recently, McDaniel et al. (Science 262:1546- 1550 (1995)) have developed a Streptomyces host-vector system for efficient construction and expression of recombinant PKSs. Hutchinson (Bio/Technolo 12:375-308 (1994)) reviewed targeted mutation of specific biosynthetic genes and suggested that microbial isolates can be screened by DNA hybridization for genes associated with known pharmacologically active agents so as to provide new metabolites or increased yields of metabolites already being produced. In particular, that review focuses on polyketide synthase and pathways to aminoglycoside and oligopeptide antibiotics.

The recursive sequence recombination techniques of the instant invention can be used to generate modified enzymes that produce novel polyketides without such detailed analytical effort. The availability of the PKS genes on plasmids and the existence of E. coli- Streptomyces shuttle vectors (Wehmeier Gene 165:149-150 (1995)) makes the process of recursive sequence recombination especially attractive by the techniques described above. Techniques for selection of antibiotic producing organisms can be used as described above; additionally, in some embodiments screening for a particular desired polyketide activity or compound is preferable.

8.14.5 ISOPRENOIDS

Isoprenoids result from cyclization of farnesyl pyrophosphate by sesquiterpene synthases. The diversity of isoprenoids is generated not by the backbone, but by control of cyclization. Cloned examples of isoprenoid synthesis genes include trichodiene synthase from *Fusarium sporotrichioides*, pentalene synthase from *Streptomyces*, aristolochene synthase from *Penicillium roquefortii*, and epi-aristolochene synthase from *N. tabacum* (Cane, D.E. (1995). Isoprenoid antibiotics, pages 633-655, in "Genetics and Biochemistry of Antibiotic Production" edited by Vining, L.C. & Stuttard, C., published by Butterworth-Heinemann). Recursive sequence recombination of sesquiterpene synthases will be of use both in allowing expression of these enzymes in heterologous hosts (such as plants and industrial microbial strains) and in alteration of enzymes to change the cyclized product



Modifications of the original peptide include (in lantibiotics) dehydration of serine and threonine, condensation of dehydroamino acids with cysteine, or simple N- and C-terminal blocking (microcins). For ribosomally made antibiotics both the peptide-encoding sequence and the modifying enzymes may have their expression levels modified by recursive sequence recombination. Again, this will lead to both increased levels of antibiotic synthesis, and by modulation of the levels of the modifying enzymes (and the sequence of the ribosomally synthesized peptide itself) novel antibiotics.

Screening can be done as for other antibiotics as described above, including competition with a sensitive (or even initially insensitive) microbial species. Use of competing bacteria that have resistances to the antibiotic being produced will select strongly either for greatly elevated levels of that antibiotic (so that it swamps out the resistance mechanism) or for novel derivatives of that antibiotic that are not neutralized by the resistance mechanism.

8.14.7 POLYMERS

Several examples of metabolic engineering to produce biopolymers have been reported, including the production of the biodegradable plastic polyhydroxybutyrate (PHB), and the polysaccharide xanthan gum. For a review, see Cameron et al. *Applied Biochem. Biotech.* 38:105-140 (1993). Genes for these pathways have been cloned, making them excellent candidates for the recursive sequence recombination techniques described above. Expression of such evolved genes in a commercially viable host such as *E. coli* is an especially attractive application of this technology.

Examples of starting materials for recursive sequence recombination include but are not limited to genes from bacteria such as *Alcaligenes*, *Zoogloea*, *Rhizobium*, *Bacillus*, and *Azobacter*, which produce polyhydroxyalkanoates (PHAs) such as polyhydroxybutyrate (PHB) intracellularly as energy reserve materials in response to stress. Genes from *Alcaligenes eutrophus* that encode enzymes catalyzing the conversion of acetoacetyl CoA to PHB have been transferred both to *E. coli* and to the plant *Arabidopsis thaliana* (Poirier et al. *Science* 256:520-523 (1992)). Two of these genes (*phbB* and *phbC*, encoding

recombination.

The recursive sequence recombination techniques of the invention as described above can be used to evolve one or more carotenoid synthesis genes in a desired host without the need for analysis of regulatory mechanisms. Since carotenoids are colored, a colorimetric assay in microtiter plates, or even on growth media plates, can be used for screening for increased production.

In addition to increasing expression of carotenoids, carotenogenic biosynthetic pathways have the potential to produce a wide diversity of carotenoids, as the enzymes involved appear to be specific for the type of reaction they will catalyze, but not for the substrate that they modify. For example, two enzymes from the marine bacterium *Agrobacterium aurantiacum* (CrtW and CrtZ) synthesize six different ketocarotenoids from beta-carotene (Misawa et al. J. Bact. 177:6576-6584 (1995)). This relaxed substrate specificity means that a diversity of substrates can be transformed into an even greater diversity of products. Introduction of foreign carotenoid genes into a cell can lead to novel and functional carotenoid-protein complexes, for example in photosynthetic complexes (Hunter et al. J.Bact. 176:3692- 3697 (1994)). Thus, the deliberate recombination of enzymes through the recursive sequence recombination techniques of the invention is likely to generate novel compounds. Screening for such compounds can be accomplished, for example, by the cell competition/survival techniques discussed above and by a colorimetric assay for pigmented compounds.

Another method of identifying new compounds is to use standard analytical techniques such as mass spectroscopy, nuclear magnetic resonance, high performance liquid chromatography, etc. Recombinant microorganisms can be pooled and extracts or media supernatants assayed from these pools. Any positive pool can then be subdivided and the procedure repeated until the single positive is identified ("sib-selection").

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Nevertheless, bacterially produced indigo is not currently an economic proposition. The recursive sequence recombination techniques of the instant invention could be used to optimize indigo synthesizing enzyme expression levels and catalytic activities, leading to increased indigo production, thereby making the process commercially viable and reducing the environmental impact of indigo manufacture. Screening for increased indigo production can be done by colorimetric assays of cultures in microtiter plates.

Amino acids of particular commercial importance include but are not limited to phenylalanine, monosodium glutamate, glycine, lysine, threonine, tryptophan and methionine. Backman et al. (Ann. NY Acad. Sci. 589:16-24 (1990)) disclosed the enhanced production of phenylalanine in *E. coli* via a systematic and downstream strategy covering organism selection, optimization of biosynthetic capacity, and development of fermentation and recovery processes. As described in Simpson et al. (Biochem Soc Trans, 23:381-387 (1995)), current work in the field of amino acid production is focused on understanding the regulation of these pathways in great molecular detail.

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8.15 TEST FOR RESISTANCE TO DRUGS

8.15.1 FIND DRUGS THAT INDUCE RESISTANCE SLOWLY

A similar strategy can be used to simulate viral acquisition of drug resistance. The object is to identify drugs for which resistance can be acquired only slowly, if at all. The viruses to be evolved are those that cause infections in humans for which at least modestly effective drugs are available. Substrates for recombination can come from induced mutants, natural variants of the same viral strain or different viruses. If the target of the drug is known (e.g., nucleotide analogs which inhibit the reverse transcriptase gene of HIV), focused libraries containing variants of the target gene can be produced. Recombination of a viral genome with a library of fragments is usually performed in vitro. However, in situations in which the library of fragments constitutes variants of viral genomes or fragments that can be encompassed in such genomes, recombination can also be performed in vivo, e.g., by transfecting cells with multiple substrate copies (see Section V). For screening, recombinant viral genomes are introduced into host cells susceptible to infection by the virus and the cells are exposed to a drug effective against the virus (initially at low concentration). The cells can be spun to remove any noninfected virus. After a period of infection, progeny viruses can be collected from the culture medium, the progeny viruses being enriched for viruses that have acquired at least partial resistance to the drug. Alternatively, virally infected cells can be plated in a soft agar lawn and resistant viruses isolated from plaques. Plaque size provides some indication of the degree of viral resistance.


Progeny viruses surviving screening are subject to additional rounds of recombination and screening at increased stringency until a predetermined level of drug resistance has been acquired. The predetermined level of drug resistance may reflect the maximum dosage of a drug practical to administer to a patient without intolerable side effects. The analysis is particularly valuable for investigating acquisition of resistance to various combination of drugs, such as the growing list of approved anti-HIV drugs (e.g., AZT, ddI, ddC, d4T, TIBO 82150, nevirapine, 3TC, crixivan and ritonavir).



THE FUTURE OF THE PAPER

After induction of recombination between YACs and natural yeast chromosomes, YACs are often eliminated by selecting against a negative selection marker on the YACs. For example, YACs containing the marker URA3 can be selected against by propagation on media containing 5-fluoro orotic acid. Any exogenous or altered genetic material that remains is contained within natural yeast chromosomes. Optionally, further rounds of recombination between natural yeast chromosomes can be performed after elimination of YACs. Optionally, the same or different library of YACs can be transformed into the cells, and the above steps repeated. By recursively repeating this process, the diversity of the population is increased prior to screening.

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The starting population of DNA fragments show sequence similarity with each other but differ as a result of for example, induced, allelic or species diversity. Often DNA fragments are known or suspected to encode multiple genes that function in a common pathway.

The fragments are cloned into a Yac and transformed into yeast, typically with positive selection for transformants. The transformants are induced to sporulate, as a result of which chromosomes undergo meiosis. The cells are then mated. Most of the resulting diploid cells now carry two YACs each having a different insert. These are again induced to sporulate and mated. The resulting cells harbor YACs of recombined sequence. The cells can then be screened or selected for a desired property. Typically, such selection occurs in the yeast strain used for stochastic &/or non-stochastic mutagenesis. However, if fragments being stochastic &/or non-stochastic mutagenized are not expressed in yeast, YACs can be isolated and transferred to an appropriate cell type in which they are expressed for screening. Examples of such properties include the synthesis or degradation of a desired compound, increased secretion of a desired gene product, or other detectable phenotype.

Preferably, the YAC library is transformed into haploid a and haploid a cells. These cells are then induced to mate with each other, i.e., they are pooled and induced to mate by growth on rich medium. The diploid cells, each carrying two YACs, are then transferred to sporulation medium. During sporulation, the cells undergo meiosis, and homologous chromosomes recombine. In this case, the genes harbored in the YACs will recombine, diversifying their sequences. The resulting haploid aconidia are then liberated

from the asci by enzymatic degradation of the asci wall or other available means and the pooled liberated haploid aco-spores are induced to mate by transfer to rich medium. This process is repeated for several cycles to increase the diversity of the DNA cloned into the YACs. The resulting population of yeast cells, preferably in the haploid state, are either screened for improved properties, or the diversified DNA is delivered to another host cell or organism for screening.

Cells surviving selection/screening are subjected to successive cycles of pooling, sporulation, mating and selection/screening until the desired phenotype has been observed. Recombination can be achieved simply by transferring cells from rich medium to carbon and nitrogen limited medium to induce sporulation, and then returning the spores to rich media to induce mating. Asci can be lysed to stimulate mating of spores originating from different asci.

After YACs have been evolved to encode a desired property they can be transferred to other cell types. Transfer can be by protoplast fusion, or retransformation with isolated DNA. For example, transfer of YACs from yeast to mammalian cells is discussed by Monaco & Larin, Trends in BiotechnoloSy 12, 280-286 (1994); Montoliu et al., Reprod. Fertil. Dev. 6, 577-84 (1994); Lamb et al., Curr. Opin. Genet. Dev. 5, 342-8 (1995). An exemplary scheme for stochastic &/or non-stochastic mutagenesis a YAC fragment library in yeast is shown herein. A library of YAC fragments representing genetic variants are transformed into yeast that have diploid endogenous chromosomes. The transformed yeast continue to have diploid endogenous chromosomes, plus a single YAC. The yeast are induced to undergo meiosis and sporulate. The spores contain haploid genomes and are selected for those which contain a YAC, using the YAC selective marker. The spores are induced to mate generating diploid cells. The diploid cells now contain two YACs bearing different inserts as well as diploid endogenous chromosomes. The cells are again induced to undergo meiosis and sporulate. during meiosis, recombination occurs between the YAC inserts, and recombinant YACs are segregated to ascocytes. Some ascocytes thus contain haploid endogenous chromosomes plus a YAC chromosome with a recombinant insert. The ascocytes mature to spores, which can mate

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again generating diploid cells. Some diploid cells now possess a diploid complement of endogenous chromosomes plus two recombinant YACs. These cells can then be taken through further cycles of meiosis, sporulation and mating. In each cycle, further recombination occurs between YAC inserts and further recombinant forms of inserts are generated. After one or several cycles of recombination has occurred, cells can be tested for acquisition of a desired property. Further cycles of recombination, followed by selection, can then be performed in similar fashion.

8.15.4 IN VIVO STOCHASTIC &/OR NON-STOCHASTIC MUTAGENESIS OF GENES BY THE RECURSIVE MATING OF YEAST CELLS HARBORING HOMOLOGOUS GENES IN IDENTICAL LOCI

A goal of DNA stochastic &/or non-stochastic mutagenesis is to mimic and expand the combinatorial capabilities of sexual recombination. In vitro DNA stochastic &/or non-stochastic mutagenesis succeeds in this process. However, by changing the mechanism of recombination and altering the conditions under which recombination occurs, naturally in vitro recombination methods may jeopardize intrinsic information in a DNA sequence that renders it "evolvable." Stochastic &/or non-stochastic mutagenesis in vivo by employing the natural crossing over mechanisms that occur during meiosis may access inherent natural sequence information and provide a means of creating higher quality stochastic &/or non-stochastic mutagenized libraries. Described here is a method for the in vivo stochastic &/or non-stochastic mutagenesis of DNA that utilizes the natural mechanisms of meiotic recombination and provides an alternative method for DNA stochastic &/or non-stochastic mutagenesis.

The basic strategy is to clone genes to be stochastic &/or non-stochastic mutagenized into identical loci within the haploid genome of yeast. The haploid cells are then recursively induced to mate and to sporulate. The process subjects the cloned genes to recursive recombination during recursive cycles of meiosis. The resulting stochastic &/or non-stochastic mutagenized genes are then screened in situ or isolated and screened under different conditions.

For example, if one wished to reassemble a family of five lipase genes, the following provides a means of doing so in vivo.

The open reading frame of each lipase is amplified by the PCR such that each ORF is flanked by identical 3' and 5' sequences. The 5' flanking sequence is identical to a region within the 5' coding sequence of the *S. cerevisiae* ura 3 gene and the 3' flanking sequence is identical to a region within the 3' of the ura 3 gene. The flanking sequences are chosen such that homologous recombination of the PCR product with the ura 3 gene results in the incorporation of the lipase gene and the disruption of the ura 3 ORF. Both *S. cerevisiae* a and haploid cells are then transformed with each of the PCR amplified lipase ORFs, and cells having incorporated a lipase gene into the ura 3 locus are selected by growth on 5 fluoro orotic acid (5FOA is lethal to cells expressing functional URA3). The result is 10 cell types, two different mating types each harboring one of the five lipase genes in the disrupted ura 3 locus. These cells are then pooled and grown under conditions where mating between the a and cells are favored, e.g. in rich medium. Mating results in a combinatorial mixture of diploid cells having all 32 possible combinations of lipase genes in the two ura 3 loci. The cells are then induced to sporulate by growth under carbon and nitrogen limited conditions. During sporulation the diploid cells undergo meiosis to form four (two a and two) haploid ascospores housed in an ascus.

During meiosis II of the sporulation process sister chromatids align and crossover. The lipase genes cloned into the ura 3 loci will also align and recombine. Thus the resulting haploid ascospores will represent a library of cells each harboring a different possible chimeric lipase gene, each a unique result of the meiotic recombination of the two lipase genes in the original diploid cell. The walls of asci are degraded by treatment with zymolase to liberate and allow the mixing of the individual ascospores. This mixture is then grown under conditions that promote the mating of the a and h aloid cells. It is important to liberate the individual ascospores, since mating will otherwise occur between the ascospores within an ascus.

Mixing of the haploid cells allows recombination between more than two lipase genes, enabling "poolwise recombination." Mating brings together new combinations of chimeric genes that can then undergo recombination upon sporulation. The cells are recursively cycled through sporulation, ascospore mixing, and mating until sufficient diversity has been generated by the recursive pairwise recombination of the five lipase genes. The individual chimeric lipase genes either can be screened directly in the haploid yeast cells or transferred to an appropriate expression host.

The process is described above for lipases and yeast; however, any sexual organisms into which genes can be directed can be employed, and any genes, of course, could be substituted for lipases. This process is analogous to the method of stochastic &/or non-stochastic mutagenesis whole genomes by recursive pairwise mating. The diversity, however, in the whole genome case is distributed throughout the host genome rather than localized to specific loci.

8.15.5 USING YACs TO CLONE UNLINKED GENES BUT FUNCTIONALLY IMPORTANT GENES FROM ONE SPECIES INTO ANOTHER

Stochastic &/or non-stochastic mutagenesis of YACs is particularly amenable to transfer of unlinked but functionally related genes from one species to another, particularly where such genes have not been identified. Such is the case for several commercially important natural products, such as taxol. Transfer of the genes in the metabolic pathway to a different organism is often desirable because organisms naturally producing such compounds are not well suited for mass culturing.

Clusters of such genes can be isolated by cloning a total genomic library of DNA from an organisms producing a useful compound into a YAC library. The YAC library is then transformed into yeast. The yeast is sporulated and mated such that recombination occurs between YACs and/or between YACs and natural yeast chromosomes.

Selection/screening is then performed for expression of the desired collection of genes. If the genes encode a biosynthetic pathway, expression can be detected from the appearance of product of the pathway. Production of individual enzymes in the pathway, or intermediates of the final expression product or capacity of cells to metabolize such intermediates indicates partial acquisition of the synthetic pathway. The original library or a different library can be introduced into cells surviving/selection screening, and further rounds of recombination and selection/screening can be performed until the end product of the desired metabolic pathway is produced.

8.15.6 YAC-YAC STOCHASTIC &/OR NON-STOCHASTIC MUTAGENESIS

If a phenotype of interest can be isolated to a single stretch of genomic DNA less than 2 megabases in length, it can be cloned into a YAC and replicated in *S. cerevisiae*. The cloning of similar stretches of DNA from related hosts into an identical YAC results in a population of yeast cells each harboring a YAC having a homologous insert effecting a desired phenotype. The recursive breeding of these yeast cells allows the homologous regions of these YACs to recombine during meiosis, allowing genes, pathways, and clusters to recombine during each cycle of meiosis. After several cycles of mating and segregation, the YAC inserts are well stochastic &/or non-stochastic mutagenized. The now very diverse yeast library could then be screened for phenotypic improvements resulting from the stochastic &/or non-stochastic mutagenesis of the YAC inserts.

8.15.7 YAC-CHROMOSOME STOCHASTIC &/OR NON-STOCHASTIC MUTAGENESIS

“Mitotic” recombination occurs during cell division and results from the recombination of genes during replication. This type of recombination is not limited to that between sister chromatids and can be enhanced by agents that induce recombination machinery, such as nicking chemicals and ultraviolet irradiation. Since it is often difficult to directly mate across a species barrier, it is possible to induce the recombination of

homologous genes originating from different species by providing the target genes to a desired host organism as a YAC library. The genes harbored in this library are then induced to recombine with homologous genes on the host chromosome by enhanced mitotic recombination. This process is carried out recursively to generate a library of diverse organisms and then screened for those having the desired phenotypic improvements. The improved subpopulation is then mated recursively as above to identify new strains having accumulated multiple useful genetic alterations.

8.15.8 ACCUMULATION OF MULTIPLE YACs HARBORING USEFUL GENES

The accumulation of multiple unlinked genes that are required for the acquisition or improvement of a given phenotype can be accomplished by the stochastic &/or non-stochastic mutagenesis of YAC libraries. Genomic DNA from organisms having desired phenotypes, such as ethanol tolerance, thermotolerance, and the ability to ferment pentose sugars are pooled, fragmented and cloned into several different YAC vectors, each having a different selective marker (his, ura, ade, etc). *S. cerevisiae* are transformed with these libraries, and selected for their presence (using selective media i.e uracil dropout media for the YAC containing the ura3 selective marker) and then screened for having acquired or improved a desired phenotype.

Surviving cells are pooled, mated recursively, and selected for the accumulation of multiple YACs (by propagation in medium with multiple nutritional dropouts). Cells that acquire multiple YACs harboring useful genomic inserts are identified by further screening. Optimized strains can be used directly, however, due to the burden a YAC may pose to a cell, the relevant YAC inserts can be minimized, subcloned, and recombined into the host chromosome, to generate a more stable production strain.

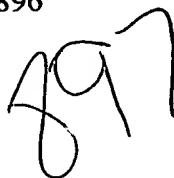
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8.15.9 CHOICE OF HOST SSF ORGANISM

One example use for the present invention is to create an improved yeast for the production of ethanol from lignocellulosic biomass. Specifically, a yeast strain with improved ethanol tolerance and thermostability/thermotolerance is desirable. Parent yeast strains known for good behavior in a Simultaneous Saccharification and Fermentation (SSF) process are identified. These strains are combined with others known to possess ethanol," tolerance and/or thermostability.

S. cerevisiae is highly amenable to development for optimized SSF processes. It inherently possesses several traits for this use, including the ability to import and ferment a variety of sugars such as sucrose, glucose, galactose, maltose and maltotriose. Also, yeast has the capability to flocculate, enabling recovery of the yeast biomass at the end of a fermentation cycle, and allowing its re-use in subsequent bioprocesses. This is an important property in that it optimizes the use of nutrients in the growth medium. *S. cerevisiae* is also highly amenable to laboratory manipulation, has highly characterized genetics and possesses a sexual reproductive cycle. *S. cerevisiae* may be grown under either aerobic or anaerobic conditions, in contrast to some other potential SSF organisms that are strict anaerobes (e.g. *Clostridium* spp.), making them very difficult to handle in the laboratory. *S. cerevisiae* are also "generally regarded as safe" ("GRAS"), and, due to its widespread use for the production of important comestibles for the general public (e.g. beer, wine, bread, etc), is generally familiar and well known. *S. cerevisiae* is commonly used in fermentative processes, and the familiarity in its handling by fermentation experts eases the introduction of novel improved yeast strains into the industrial setting. *S. cerevisiae* strains that previously have been identified as particularly good SSF organisms, for example, *S. cerevisiae* D₅A (ATCC200062) (South CR and Lynd LR. (1994) Appl. Biochem. Biotechnol. 45/46: 467-481; Ranatunga TD et al. (1997) Biotechnol. Lett. 19:1125-1127) can be used for starting materials. In addition, other industrially used *S. cerevisiae* strains are optionally used as host strains, particularly those showing desirable fermentative characteristics, such as *S. cerevisiae* Y567 (ATCC24858) (Sitton OC et al. (1979) Process Biochem. 14(9): 7-10; Sitton OC et al. (1981) Adv. Biotechnol. 2: 231-237; McMurrough I et al. (1971) Folia Microbiol. 16: 346-349) and *S. cerevisiae* ACA 174 (ATCC 60868) (Benitez T et al. (1983) Appl. Environ. Microbiol. 45: 1429-1436;



Chem. Eng. J. 50: B I 7-B22, 1992), which have been shown to have desirable traits for large-scale fermentation.

8.15.10 CHOICE OF ETHANOL TOLERANT STRAINS

Many strains of *S. cerevisiae* have been isolated from high-ethanol environments, and have survived in the ethanol-rich environment by adaptive evolution. For example, strains from Sherry wine aging ("Flor" strains) have evolved highly functional mitochondria to enable their survival in a high-ethanol environment. It has been shown that transfer of these wine yeast mitochondria to other strains increases the recipient's resistance to high ethanol concentration, as well as thermotolerance (Jimenez, J. and Benitez, T (1988) Curr. Genet. 13: 461-469). There are several flor strains deposited in the ATCC, for example *S. cerevisiae* MY91 (ATCC 201301), MY138 (ATCC 201302), C5 (ATCC 201298), ET7 (ATCC 201299), LA6 (ATCC 201300), OSB21 (ATCC 201303), F23 (*S. globosus* ATCC 90920). Also, several flor strains of *S. uvarum* and *Torulaspora pretoriensis* have been deposited. Other ethanol-tolerant wine strains include *S. cerevisiae* ACA 174 (ATCC 60868), 15% ethanol, and *S. cerevisiae* A54 (ATCC 90921), isolated from wine containing 18% (v/v) ethanol, and NRCC 202036 (ATCC 46534), also a wine yeast. Other *S. cerevisiae* ethanologens that additionally exhibit enhanced ethanol tolerance include ATCC 24858, ATCC 24858, G 3706 (ATCC 42594), NRRL Y-265 (ATCC 60593), and ATCC 24845 - ATCC 24860. A strain of *S. pastorianus* (*S. carlsbergensis* ATCC 2345) has high ethanol-tolerance (13% v/v). *S. cerevisiae* Sa28 (ATCC 26603), from Jamaican cane juice sample, produces high levels of alcohol from molasses, is sugar tolerant, and produces ethanol from wood acid hydrolyzate. Several of the listed strains, as well as additional strains can be used as starting materials for breeding ethanol tolerance.

8.15.11 CHOICE OF TEMPERATURE TOLERANT STRAINS

A few temperature tolerant strains have been reported, including the highly flocculent strain *S. pastorianus* SA 23 (*S. carlsbergensis* ATCC 26602), which produces

ethanol at elevated temperatures, and. *S. cerevisiae* Kyokai 7 (*S. sake*, ATCC 26422), a sake yeast tolerant to brief heat and oxidative stress. Ballesteros et al ((1991) Appl. Biochem. Biotechnol. 28/29: 307-315) examined 27 strains of yeast for their ability to grow and ferment glucose in the 32-45°C temperature range, including *Saccharomyces*, *Kluyveromyces* and *Candida* spp. Of these, the best thermotolerant clones were *Kluyveromyces marxianus* LG and *Kluyveromyces fragilis* 2671 (Ballesteros et al (1993) Appl. Biochem. Biotechnol. 39/40: 201-211). *S. cerevisiae*-pretoriensis FDHI was somewhat thermotolerant, however was poor in ethanol tolerance. Recursive recombination of this strain with others that display ethanol tolerance can be used to acquire the thermotolerant characteristics of the strain in progeny which also display ethanol tolerance. *Candida acidothermophilum* (*Issatchenlaa orientalis*, ATCC 203 8 1) is a good SSF strain that also exhibits improved performance in ethanol production from lignocellulosic biomass at higher SSF temperatures than *S. cerevisiae* D₅A (Kadam, KL, Schmidt, SL (1997) Appl. Microbial. Biotechnol. 48: 709-713). This strain can also be a genetic contributor to an improved SSF strain.

8.15.12 STOCHASTIC &/OR NON-STOCHASTIC MUTAGENESIS OF STRAINS

In those instances where strains are highly related, a recursive mating strategy may be pursued. For example, a population of haploid *S. cerevisiae* (a and) a r e mutagenized and screened for improved EtOH or thermal tolerance. The improved haploid subpopulation are mixed together and mated as a pool and induced to sporulate. The resulting haploid spores are freed by degrading the asci wall and mixed. The freed spores are then induced to mate and sporulate recursively. This process is repeated a sufficient number of times to generate all possible mutant combinations. The whole genome stochastic &/or non-stochastic mutagenized population (haploid) is then screened for further EtOH or thermal tolerance.

When strains are not sufficiently related for recursive mating, formats based on protoplast fusion may be employed. Recursive and poolwise protoplast fusion can be performed to generate chimeric populations of diverse parental strains. The resultant pool

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Simple selection schemes for identification of thermal tolerant and ethanol tolerant strains are available and, in this case, are based on those previously designed to identify potentially useful SSF strains. Selection of ethanol tolerance is performed by exposing the population to ethanol, then plating the population and looking for growth. Colonies capable of growing after exposure to ethanol can be re-exposed to a higher concentration of ethanol and the cycle repeated until the most tolerant strains are selected. In order to discern strains possessing heritable ethanol tolerance from with temporarily acquired adaptations, these cycles may be punctuated with cycles of growth in the absence of selection (e.g. no ethanol).

Alternatively, the mixed population can be grown directly at increasing concentrations of ethanol, and the most tolerant strains enriched (Aguilera and Benitez, 1986, Arch Microbiol 4:337-44). For example this enrichment could be carried out in a chemostat or turbidostat. Similar selections can be developed for thermal tolerance, in which strains are identified by their ability to grow after a heat treatment, or directly for growth at elevated temperatures (Ballesteros et al., 1991, Applied Biochem and Biotech, 28:307-315). The best strains identified by these selections will be assayed more thoroughly in subsequent screens for ethanol, thermal tolerance or other properties of interest.

In one aspect, organisms having increased ethanol tolerance are selected for. A population of natural *S. cerevisiae* isolates are mutagenized. This population is then grown under fermentor conditions under low initial ethanol concentrations. Once the culture has reached saturation, the culture is diluted into fresh medium having a slightly higher ethanol content. This process of successive dilution into medium of incrementally increasing ethanol concentration is continued until a threshold of ethanol tolerance is reached. The surviving mutant population having the highest ethanol tolerance are then pooled and their genomes recombined by any method noted herein. Enrichment could also be achieved by a continuous culture in a chemostat or turbidostat in which temperature or ethanol concentrations are progressively elevated. The resulting stochastic &/or non-stochastic mutagenized population are then exposed once again to the enrichment strategy

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Strains showing viability in initial selections are assayed more quantitatively for improvements in the desired properties before being restochastic &/or non-stochastic mutagenized with other strains.

8.15.15 DEVELOPMENT OF A YEAST STRAIN CAPABLE OF CONVERTING CELLULOSE TO MONOMERIC SUGARS

Once a strain of yeast exhibiting thermotolerance and ethanol tolerance is developed, the degradation of cellulose to monomeric sugars is provided by the inclusion to the host strain of an efficient cellulase degradation pathway.

Additional desirable characteristic can be useful to enhance the production of ethanol by the host. For example, inclusion of heterologous enzymes and pathways that broaden the substrate sugar range may be performed. "Tuning" of the strain can be accomplished by the addition of various other traits, or the restoration of certain endogenous traits that are desirable, but lost during the recombination procedures.

8.15.16 CONFERRING OF CELLULASE ACTIVITY

A vast number of cellulases and cellulase degradation systems have been characterized from fungi, bacteria and yeast (see reviews by Beguin, P and Aubert, J-P (1994) FEMS Microbial. Rev. 13: 25-58; Ohima, K. et al. (1997) Biotechnol. Genet. Eng. Rev. 14: 365-414). An enzymatic pathway required for efficient saccharification of cellulose involves the synergistic action of endoglucanases (endo-1,4- β -D-glucanases, EC 3.2.1.4), exocellobiohydrolases (exo-1,4- β -D-glucanases, EC 3.2.1.91), and β -glucosidases (cellobiases, 1,4- β -D-glucanases EC 3.2.1.21). The heterologous production of cellulase enzymes in the ethanologen would enable the saccharification of cellulose, producing monomeric sugars that may be used by the organism for ethanol production. There are several advantages to the heterologous expression of a functional cellulase pathway in the ethanologen. For example, the SSF process would eliminate the need for a separate bioprocess step for saccharification, and would ameliorate end-product inhibition of cellulase enzymes by accumulated intermediate and product sugars.

Naturally occurring cellulase pathways are inserted into the ethanologen, or one may choose to use custom improved "hybrid" cellulase pathways, employing the coordinate action of cellulases derived from different natural sources, including thermophiles.

Several cellulases from non-Saccharomyces have been produced and secreted from this organism successfully, including bacterial, fungal, and yeast enzymes, for example T. reesei CBH I ((Shoemaker (1994), in "The Cellulase System of Trichoderma reesei: Trichoderma strain improvement and Expression of Trichoderma cellulases in Yeast," Online, Pinner, UK, 593-600). It is possible to employ straightforward metabolic engineering techniques to engender cellulase activity in Saccharomyces. Also, yeast have been forced to acquire elements of cellulose degradation pathways by protoplast fusion (e.g. intergeneric hybrids of Saccharomyces cerevisiae and Zygosaccharomyces fermentati, a cellobiase-producing yeast, have been created (Pina A, et. al. (1986) Appl. Environ. Microbial. 51: 995- 1003). In general, any cellulase component enzyme that

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derives from a closely related yeast organism could be transferred by protoplast fusion. Cellobiases produced by a somewhat broader range of yeast may be accessed by whole genome stochastic &/or non-stochastic mutagenesis in one of its many formats (e.g. whole, fragmented, YAC-based).

Optimally, the cellulase enzymes to be used should exhibit good synergy, an appropriate level of expression and secretion from the host, good specific activity (i.e. resistance to host degradation factors and enzyme modification) and stability in the desired SSF environment. An example of a hybrid cellulose degradation pathway having excellent synergy includes the following enzymes: CBH I exocellobiohydrolase of *Trichoderma reesei*, the *Acidothermus cellulolyticus* E1 endoglucanase, and the *Thermomonospora fusca* E3 exocellulase (Baker, et. al. (1998) Appl. Biochem. Biotechnol. 70-72: 395-403).

It is suggested here that these enzymes (or improved mutants thereof) be considered for use in the SSF organism, along with a cellobiase (- g l ucosidase), such as that from *Candida peltata*. Other possible cellulase systems to be considered should possess particularly good activity against crystalline cellulose, such as the *T. reesei* cellulase system (Teeri, TT, et. al. (1998) Biochem. Soc, Trans. 26: 173-178), or possess particularly good thermostability characteristics (e.g. cellulase systems from thermophilic organisms, such as *Thermomonospora fusca* (Zhang, S., et. al. (1995) Biochem.. 34: 3 3 86-3 3 5)).

A rational approach to the cloning of cellulases in the ethanologenic yeast host could be used. For example, known cellulase genes are cloned into expression cassettes utilizing *S. cerevisiae* promoter sequences, and the resultant linear fragments of DNA may be transformed into the recipient host by placing short yeast sequences at the termini to encourage site-specific integration into the genome. This is preferred to plasmidic transformation for reasons of genetic stability and maintenance of the transforming DNA.

If an entire cellulose degradative pathway were introduced, a selection could be implemented in an agar-plate-based format, and a large number of clones could be assayed

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for cellulase activity in a short period of time. For example, selection for an exocellulase may be accessible by providing a soluble oligocellulose substrate or carboxymethylcellulose (CMC) as a sole carbon source to the host, otherwise unable to grow on agar containing this sole carbon source. Clones producing active cellulase pathways would grow by virtue of their ability to produce glucose.

Alternatively, if the different cellulases were to be introduced sequentially, it would be useful to first introduce a cellobiase, enabling a selection using commercially available cellobiose as a sole carbon source. Several strains of *S. cerevisiae* that are able to grow on cellobiose have been created by introduction of a cellobiase gene (e.g. Rajoka MI, et. al. (1998) *Floia Microbiol. (Praha)* 43, 129-135; Skory, CD, et. al. (1996) *Curr. Genet.* 30, 417-422; D'Auria, S, et. al. (1996) *Appl. Biochem. Biotechnol.* 61, 157-166; Adam, AC, et. al. (1995) *Yeast* 11, 395-406; Adam, AC (1991) *Cuff. Genet.* 20, 5-8).

Subsequent transformation of this organism with CBHI exocellulase can be selected for by growth on a cellulose substrate such as carboxymethylcellulose (CMC). Finally, addition of an endoglucanase creates a yeast strain with improved crystalline degradation capacity.

8.15.17 CONFERRING OF PENTOSE SUGAR UTILIZATION

Inclusion of pentose sugar utilization pathways is an important facet to a potentially useful SSF organism. The successful expression of xylose sugar utilization pathways for ethanol production has been reported in *Saccharomyces* (e.g. Chen, ZD and Ho, NWY (1993) *Appl. Biochem. Biotechnol.* 39/40 135-147).

It would also be useful to accomplish L-arabinose substrate utilization for ethanol production in the *Saccharomyces* host. Yeast strains that utilize L-arabinose include some *Candida* and *Pichia* spp. (McMillan JD and Boynton BL (1994) *Appl. Biochem. Biotechnol.* 45-46: 569-584; Dien BS, et al. (1996) *Appl. Biochem. Biotechnol.* 57-58: 233-242). Genes necessary for arabinose fermentation in *E. coli* could also be introduced

by rational means (e.g. as has been performed previously in *Z. mobilis* (Deanda K, et. al. (1996) Appl. Environ. Microbial. 62: 4465-4470))

8.15.18 CONFERRING OF OTHER USEFUL ACTIVITIES

Several other traits that are important for optimization of an SSF strain have been shown to be transferable to *S. cerevisiae*. Like thermal tolerance, cellulase activity and pentose sugar utilization, these traits may not normally be exhibited by *Saccharomyces* (or the particular strain of *Saccharomyces* being used as a host), and may be added by genetic means.

For example, expression of human muscle acylphosphatase in *S. cerevisiae* has been suggested to increase ethanol production (Rougei, G., et. al. (1996) Biotechnol. App. Biochem. 23: 273- 278).

It can occur that evolved stress-tolerant SSF strain acquire some undesirable mutations in the course of the evolution strategy. Indeed, this is a pervasive problem in strain improvement strategies that rely on mutagenesis techniques, and can result in highly unstable or fragile production strains. It is possible to restore some of these desirable traits by rational methods such as cloning of specific genes that have been knocked out or negatively influenced in the previous rounds of strain improvement. The advantage to this approach is specificity- the offending gene may be targeted directly. The disadvantage is that it may be time-consuming and repetitious if several genes have been compromised, and it only addresses problems that have been characterized. A preferred (and more traditional) approach to the removal of undesirable/deleterious mutations is to back-cross the evolved strain to a desirable parent strain (e.g. the original "host" SSF strain). This strategy has been employed successfully throughout strain improvement where accessible (i.e. for organisms that have sexual cycles of reproduction). When lacking the advantage of a sexual process, it has been accomplished by using other methods, such as parasexual recombination or protoplast fusion.

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invariable amino acids (bits) required to describe a family of related sequences with the same function. Proteins that are more sensitive to random mutagenesis have a high information content.

Molecular biology developments, such as molecular libraries, have allowed the identification of quite a large number of variable bases, and even provide ways to select functional sequences from random libraries. In such libraries, most residues can be varied (although typically not all at the same time) depending on compensating changes in the context. Thus, while a 100 amino acid protein can contain only 2,000 different mutations, 20^{100} sequence combinations are possible.

Information density is the IC per unit length of a sequence. Active sites of enzymes tend to have a high information density. By contrast, flexible linkers of information in enzymes have a low information density.

Current methods in widespread use for creating alternative proteins in a library format are error-prone polymerase chain reactions and cassette mutagenesis, in which the specific region to be optimized is replaced with a synthetically mutagenized oligonucleotide. In both cases, a substantial number of mutant sites are generated around certain sites in the original sequence.

8.16.3.1 Error-prone PCR

Error-prone PCR uses low-fidelity polymerization conditions to introduce a low level of point mutations randomly over a long sequence. In a mixture of fragments of unknown sequence, error-prone PCR can be used to mutagenize the mixture. The published error-prone PCR protocols suffer from a low processivity of the polymerase. Therefore, the protocol is unable to result in the random mutagenesis of an average-sized gene. This inability limits the practical application of error-prone PCR. Some computer simulations have suggested that point mutagenesis alone may often be too gradual to allow the large-scale block changes that are required for continued and dramatic sequence evolution. Further, the published error-prone PCR protocols do not allow for amplification of DNA fragments greater than 0.5 to 1.0 kb, limiting their practical

application. In addition, repeated cycles of error-prone PCR can lead to an accumulation of neutral mutations with undesired results, such as affecting a protein's immunogenicity but not its binding affinity.

8.16.3.2 Oligonucleotide-Directed Mutagenesis

In oligonucleotide-directed mutagenesis, a short sequence is replaced with a synthetically mutagenized oligonucleotide. This approach does not generate combinations of distant mutations and is thus not combinatorial. The limited library size relative to the vast sequence length means that many rounds of selection are unavoidable for protein optimization. Mutagenesis with synthetic oligonucleotides requires sequencing of individual clones after each selection round followed by grouping them into families, arbitrarily choosing a single family, and reducing it to a consensus motif. Such motif is resynthesized and reinserted into a single gene followed by additional selection. This step process constitutes a statistical bottleneck, is labor intensive, and is not practical for many rounds of mutagenesis.

Error-prone PCR and oligonucleotide-directed mutagenesis are thus useful for single cycles of sequence fine tuning, but rapidly become too limiting when they are applied for multiple cycles.

Another limitation of error-prone PCR is that the rate of down-mutations grows with the information content of the sequence. As the information content, library size, and mutagenesis rate increase, the balance of down-mutations to up-mutations will statistically prevent the selection of further improvements (statistical ceiling).

8.16.3.3 Cassette Mutagenesis

In cassette mutagenesis, a sequence block of a single template is typically replaced by a (partially) randomized sequence. Therefore, the maximum information content that can be obtained is statistically limited by the number of random sequences (*i.e.*, library

size). This eliminates other sequence families which are not currently best, but which may have greater long term potential.

Also, mutagenesis with synthetic oligonucleotides requires sequencing of individual clones after each selection round. Thus, such an approach is tedious and impractical for many rounds of mutagenesis.

Thus, error-prone PCR and cassette mutagenesis are best suited, and have been widely used, for fine-tuning areas of comparatively low information content. One apparent exception is the selection of an RNA ligase ribozyme from a random library using many rounds of amplification by error-prone PCR and selection.

In nature, the evolution of most organisms occurs by natural selection and sexual reproduction. Sexual reproduction ensures mixing and combining of the genes in the offspring of the selected individuals. During meiosis, homologous chromosomes from the parents line up with one another and cross-over part way along their length, thus randomly swapping genetic material. Such swapping or shuffling of the DNA allows organisms to evolve more rapidly.

In recombination, because the inserted sequences were of proven utility in a homologous environment, the inserted sequences are likely to still have substantial information content once they are inserted into the new sequence.

8.16.3.4 Applied Molecular Evolution

The term Applied Molecular Evolution ("AME") means the application of an evolutionary design algorithm to a specific, useful goal. While many different library formats for AME have been reported for polynucleotides, peptides and proteins (phage, lacI and polysomes), none of these formats have provided for recombination by random cross-overs to deliberately create a combinatorial library.

Theoretically there are 2,000 different single mutants of a 100 amino acid protein. However, a protein of 100 amino acids has 20^{100} possible sequence combinations, a number which is too large to exhaustively explore by conventional methods. It would be

The following table shows the results of the regression analysis for the dependent variable $\ln Y$ (ln of the dependent variable) and the independent variables X_1 to X_6 (ln of the independent variables). The table is divided into two parts: the first part shows the results of the regression analysis for the dependent variable $\ln Y$ and the independent variables X_1 to X_6 , and the second part shows the results of the regression analysis for the dependent variable $\ln Y$ and the independent variables X_1 to X_6 .

Some workers in the art have utilized an *in vivo* site specific recombination system to generate hybrids of combine light chain antibody genes with heavy chain antibody genes for expression in a phage system. However, their system relies on specific sites of recombination and is limited accordingly. Simultaneous mutagenesis of antibody CDR regions in single chain antibodies (scFv) by overlapping extension and PCR have been reported.

In vivo recombination between two homologous, but truncated, insect-toxin genes on a plasmid has been reported as a method of producing a hybrid gene. The *in vivo* recombination of substantially mismatched DNA sequences in a host cell having defective mismatch repair enzymes, resulting in hybrid molecule formation has been reported.

1. The first step is to identify the problem or question that needs to be addressed. This involves understanding the context and the specific requirements of the task.

It is an object of the present invention to provide a method for generating hybrid polynucleotides encoding biologically active hybrid polypeptides with enhanced activities. In accomplishing these and other objects, there has been provided, in accordance with one aspect of the invention, a method for introducing polynucleotides into a suitable host cell and growing the host cell under conditions which produce a hybrid polynucleotide.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

The invention described herein is directed to the use of repeated cycles of reductive reassortment, recombination and selection which allow for the directed molecular evolution of highly complex linear sequences, such as DNA, RNA or proteins thorough recombination.

In vivo shuffling of molecules can be performed utilizing the natural property of cells to recombine multimers. While recombination *in vivo* has provided the major natural route to molecular diversity, genetic recombination remains a relatively complex process that involves 1) the recognition of homologies; 2) strand cleavage, strand invasion, and metabolic steps leading to the production of recombinant chiasma; and finally 3) the resolution of chiasma into discrete recombined molecules. The formation of the chiasma requires the recognition of homologous sequences.

8.16.5.1 Production of a Hybrid Polynucleotide

In a preferred embodiment, the invention relates to a method for producing a hybrid polynucleotide from at least a first polynucleotide and a second polynucleotide. The present invention can be used to produce a hybrid polynucleotide by introducing at least a first polynucleotide and a second polynucleotide which share at least one region of partial sequence homology into a suitable host cell. The regions of partial sequence homology promote processes which result in sequence reorganization producing a hybrid polynucleotide. The term "hybrid polynucleotide", as used herein, is any nucleotide sequence which results from the method of the present invention and contains sequence from at least two original polynucleotide sequences. Such hybrid polynucleotides can result from intermolecular recombination events which promote sequence integration between DNA molecules. In addition, such hybrid polynucleotides can result from intramolecular reductive reassortment processes which utilize repeated sequences to alter a nucleotide sequence within a DNA molecule.

The invention provides a means for generating hybrid polynucleotides which may encode biologically active hybrid polypeptides. In one aspect, the original polynucleotides encode biologically active polypeptides. The method of the invention produces new hybrid polypeptides by utilizing cellular processes which integrate the sequence of the original polynucleotides such that the resulting hybrid polynucleotide encodes a polypeptide demonstrating activities derived from the original biologically active polypeptides. For example, the original polynucleotides may encode a particular enzyme from different microorganisms. An enzyme encoded by a first polynucleotide from one

organism may, for example, function effectively under a particular environmental condition, *e.g.* high salinity. An enzyme encoded by a second polynucleotide from a different organism may function effectively under a different environmental condition, such as extremely high temperatures. A hybrid polynucleotide containing sequences from the first and second original polynucleotides may encode an enzyme which exhibits characteristics of both enzymes encoded by the original polynucleotides. Thus, the enzyme encoded by the hybrid polynucleotide may function effectively under environmental conditions shared by each of the enzymes encoded by the first and second polynucleotides, *e.g.*, high salinity and extreme temperatures.

8.16.5.1.1 Encoded Enzymes

Enzymes encoded by the original polynucleotides of the invention include, but are not limited to; oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. A hybrid polypeptide resulting from the method of the invention may exhibit specialized enzyme activity not displayed in the original enzymes. For example, following recombination and/or reductive reassortment of polynucleotides encoding hydrolase activities, the resulting hybrid polypeptide encoded by a hybrid polynucleotide can be screened for specialized hydrolase activities obtained from each of the original enzymes, *i.e.* the type of bond on which the hydrolase acts and the temperature at which the hydrolase functions. Thus, for example, the hydrolase may be screened to ascertain those chemical functionalities which distinguish the hybrid hydrolase from the original hydrolyases, such as: (a) amide (peptide bonds), *i.e.* proteases; (b) ester bonds, *i.e.* esterases and lipases; (c) acetals, *i.e.*, glycosidases and, for example, the temperature, pH or salt concentration at which the hybrid polypeptide functions.

8.16.5.1.2 Sources Of The Original Polynucleotides

Sources of the original polynucleotides may be isolated from individual organisms ("isolates"), collections of organisms that have been grown in defined media ("enrichment cultures"), or, most preferably, uncultivated organisms ("environmental samples"). The

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For example, gene libraries generated from one or more uncultivated microorganisms are screened for an activity of interest. Potential pathways encoding bioactive molecules of interest are first captured in prokaryotic cells in the form of gene expression libraries. Polynucleotides encoding activities of interest are isolated from such libraries and introduced into a host cell. The host cell is grown under conditions which promote recombination and/or reductive reassortment creating potentially active biomolecules with novel or enhanced activities.

The microorganisms from which the polynucleotide may be prepared include prokaryotic microorganisms, such as Eubacteria and Archaeobacteria, and lower eukaryotic microorganisms such as fungi, some algae and protozoa. Polynucleotides may be isolated from environmental samples in which case the nucleic acid may be recovered without culturing of an organism or recovered from one or more cultured organisms. In one aspect, such microorganisms may be extremophiles, such as hyperthermophiles, psychrophiles, psychrotrophs, halophiles, barophiles and acidophiles. Polynucleotides encoding enzymes isolated from extremophilic microorganisms are particularly preferred. Such enzymes may function at temperatures above 100°C in terrestrial hot springs and deep sea thermal vents, at temperatures below 0°C in arctic waters, in the saturated salt environment of the Dead

Sea, at pH values around 0 in coal deposits and geothermal sulfur-rich springs, or at pH values greater than 11 in sewage sludge. For example, several esterases and lipases cloned and expressed from extremophilic organisms show high activity throughout a wide range of temperatures and pHs.

8.16.5.1.3 Suitable Host Cells

Polynucleotides selected and isolated as hereinabove described are introduced into a suitable host cell. A suitable host cell is any cell which is capable of promoting recombination and/or reductive reassortment. The selected polynucleotides are preferably already in a vector which includes appropriate control sequences. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or preferably, the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli*, *Streptomyces*, *Salmonella typhimurium*; fungal cells, such as yeast; insect cells such as *Drosophila S2* and *Spodoptera Sf9*; animal cells such as CHO, COS or Bowes melanoma; adenoviruses; and plant cells. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

8.16.5.1.3.1 Mammalian Cell Culture Systems

With particular references to various mammalian cell culture systems that can be employed to express recombinant protein, examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA

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sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

Host cells containing the polynucleotides of interest can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. The clones which are identified as having the specified enzyme activity may then be sequenced to identify the polynucleotide sequence encoding an enzyme having the enhanced activity.

8.16.5.1.4 Generation Of Polynucleotides Encoding Biochemical Pathways

In another aspect, it is envisioned the method of the present invention can be used to generate novel polynucleotides encoding biochemical pathways from one or more operons or gene clusters or portions thereof. For example, bacteria and many eukaryotes have a coordinated mechanism for regulating genes whose products are involved in related processes. The genes are clustered, in structures referred to as "gene clusters," on a single chromosome and are transcribed together under the control of a single regulatory sequence, including a single promoter which initiates transcription of the entire cluster. Thus, a gene cluster is a group of adjacent genes that are either identical or related, usually as to their function. An example of a biochemical pathway encoded by gene clusters are polyketides. Polyketides are molecules which are an extremely rich source of bioactivities, including antibiotics (such as tetracyclines and erythromycin), anti-cancer agents (daunomycin), immunosuppressants (FK506 and rapamycin), and veterinary products (monensin). Many polyketides (produced by polyketide synthases) are valuable as therapeutic agents. Polyketide synthases are multifunctional enzymes that catalyze the biosynthesis of an enormous variety of carbon chains differing in length and patterns of functionality and cyclization. Polyketide synthase genes fall into gene clusters and at least one type (designated type I) of polyketide synthases have large size genes and enzymes, complicating genetic manipulation and *in vitro* studies of these genes/proteins.

The ability to select and combine desired components from a library of polyketides, or fragments thereof, and postpolyketide biosynthesis genes for generation of

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Preferably, gene cluster DNA can be isolated from different organisms and ligated into vectors, particularly vectors containing expression regulatory sequences which can control and regulate the production of a detectable protein or protein-related array activity from the ligated gene clusters. Use of vectors which have an exceptionally large capacity for exogenous DNA introduction are particularly appropriate for use with such gene clusters and are described by way of example herein to include the f-factor (or fertility factor) of *E. coli*. This f-factor of *E. coli* is a plasmid which affect high-frequency transfer of itself during conjugation and is ideal to achieve and stably propagate large DNA fragments, such as gene clusters from mixed microbial samples. Once ligated into an appropriate vector, two or more vectors containing different polyketide synthase gene clusters can be introduced into a suitable host cell. Regions of partial sequence homology shared by the gene clusters will promote processes which result in sequence reorganization resulting in a hybrid gene cluster. The novel hybrid gene cluster can then be screened for enhanced activities not found in the original gene clusters.

introducing at least a first polynucleotide in operable linkage and a second polynucleotide in operable linkage, said at least first polynucleotide and second polynucleotide sharing at least one region of partial sequence homology, into a suitable host cell;

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expressing a hybrid polypeptide encoded by the hybrid polynucleotide;

screening the hybrid polypeptide under conditions which promote identification of enhanced biological activity; and

isolating the a polynucleotide encoding the hybrid polypeptide.

Methods for screening for various enzyme activities are known to those of skill in the art and discussed throughout the present specification. Such methods may be employed when isolating the polypeptides and polynucleotides of the present invention.

The term "isolated" means that material is removed from its original environment (*e.g.*, the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide separated from some or all of the coexisting materials in the natural system, is isolated.

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame.

8.16.5.1.6 Expression Vectors

As representative examples of expression vectors which may be used there may be mentioned viral particles, baculovirus, phage, plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes, viral DNA (*e.g.* vaccinia, adenovirus, fowl pox virus, pseudorabies and derivatives of SV40), P1-based artificial chromosomes, yeast plasmids, yeast artificial chromosomes, and any other vectors specific for specific hosts of interest (such as bacillus, aspergillus and yeast) Thus, for example, the DNA may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors

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and promoter is well within the level of ordinary skill in the art. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers.

8.16.5.1.6.2 Selectable Marker Genes

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, *e.g.*, the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), -f factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium.

The cloning strategy permits expression via both vector driven and endogenous promoters; vector promotion may be important with expression of genes whose endogenous promoter will not function in *E. coli*.

8.16.5.1.7 Insertion Into a Vector or Plasmid

The DNA isolated or derived from microorganisms can preferably be inserted into a vector or a plasmid prior to probing for selected DNA. Such vectors or plasmids are

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Therefore, in another aspect of the present invention, novel polynucleotides can be generated by the process of reductive reassortment. The method involves the generation of constructs containing consecutive sequences (original encoding sequences), their insertion into an appropriate vector, and their subsequent introduction into an appropriate host cell. The reassortment of the individual molecular identities occurs by combinatorial processes between the consecutive sequences in the construct possessing regions of homology, or between quasi-repeated units. The reassortment process recombines and/or reduces the complexity and extent of the repeated sequences, and results in the production of novel molecular species. Various treatments may be applied to enhance the rate of reassortment. These could include treatment with ultra-violet light, or DNA damaging chemicals, and/or the use of host cell lines displaying enhanced levels of "genetic instability". Thus the reassortment process may involve homologous recombination or the natural property of quasi-repeated sequences to direct their own evolution.

8.16.5.1.9 Repeated or "Quasi-Repeated" Sequences

Repeated or "quasi-repeated" sequences play a role in genetic instability. In the present invention, "quasi-repeats" are repeats that are not restricted to their original unit structure. Quasi-repeated units can be presented as an array of sequences in a construct; consecutive units of similar sequences. Once ligated, the junctions between the consecutive sequences become essentially invisible and the quasi-repetitive nature of the resulting construct is now continuous at the molecular level. The deletion process the cell performs to reduce the complexity of the resulting construct operates between the quasi-repeated sequences. The quasi-repeated units provide a practically limitless repertoire of templates upon which slippage events can occur. The constructs containing the quasi-repeats thus effectively provide sufficient molecular elasticity that deletion (and potentially insertion) events can occur virtually anywhere within the quasi-repetitive units.

When the quasi-repeated sequences are all ligated in the same orientation, for instance head to tail or vice versa, the cell cannot distinguish individual units. Consequently, the reductive process can occur throughout the sequences. In contrast, when for example, the units are presented head to head, rather than head to tail, the

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- 1) The use of vectors only stably maintained when the construct is reduced in complexity.
 - 2) The physical recovery of shortened vectors by physical procedures. In this case, the cloning vector would be recovered using standard plasmid isolation procedures and size fractionated on either an agarose gel, or column with a low molecular weight cut off utilizing standard procedures.
 - 3) The recovery of vectors containing interrupted genes which can be selected when insert size decreases.
 - 4) The use of direct selection techniques with an expression vector and the appropriate selection.

Encoding sequences (for example, genes) from related organisms may demonstrate a high degree of homology and encode quite diverse protein products. These types of sequences are particularly useful in the present invention as quasi-repeats. However, while the examples illustrated below demonstrate the reassortment of nearly identical original encoding sequences (quasi-repeats), this process is not limited to such nearly identical repeats.

The following example demonstrates the method of the invention. Encoding nucleic acid sequences (quasi-repeats) derived from three (3) unique species are depicted. Each sequence encodes a protein with a distinct set of properties. Each of the sequences differs by a single or a few base pairs at a unique position in the sequence which are designated "A", "B" and "C". The quasi-repeated sequences are separately or collectively amplified and ligated into random assemblies such that all possible permutations and combinations are available in the population of ligated molecules. The number of quasi-repeat units can be controlled by the assembly conditions. The average number of quasi-repeated units in a construct is defined as the repetitive index (RI).

Once formed, the constructs may, or may not be size fractionated on an agarose gel according to published protocols, inserted into a cloning vector, and transfected into an

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In another aspect the present invention is directed to a method of producing recombinant proteins having biological activity by treating a sample comprising double-stranded template polynucleotides encoding a wild-type protein under conditions according to the present invention which provide for the production of hybrid or reassorted polynucleotides.

The invention also provides the use of polynucleotide shuffling to shuffle a population of viral genes (*e.g.*, capsid proteins, spike glycoproteins, polymerases, and proteases) or viral genomes (*e.g.*, paramyxoviridae, orthomyxoviridae, herpesviruses, retroviruses, reoviruses and rhinoviruses). In an embodiment, the invention provides a method for shuffling sequences encoding all or portions of immunogenic viral proteins to generate novel combinations of epitopes as well as novel epitopes created by recombination; such shuffled viral proteins may comprise epitopes or combinations of epitopes as well as novel epitopes created by recombination; such shuffled viral proteins may comprise epitopes or combinations of epitopes which are likely to arise in the natural environment as a consequence of viral evolution; (*e.g.*, such as recombination of influenza virus strains).

8.16.5.1.16 Generation of Gene Therapy Vectors and Replication-Defective Gene Therapy Constructs

The invention also provides a method suitable for shuffling polynucleotide sequences for generating gene therapy vectors and replication-defective gene therapy constructs, such as may be used for human gene therapy, including but not limited to vaccination vectors for DNA-based vaccination, as well as anti-neoplastic gene therapy and other general therapy formats.

The term "DNA shuffling" is used herein to indicate recombination between substantially homologous but non-identical sequences, in some embodiments DNA shuffling may involve crossover via non-homologous recombination, such as via cer/lox and/or flp/frt systems and the like.

The term "identical" or "identity" means that two nucleic acid sequences have the same sequence or a complementary sequence. Thus, "areas of identity" means that regions or areas of a polynucleotide or the overall polynucleotide are identical or complementary to areas of another polynucleotide or the polynucleotide.

The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (*i.e.*, is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference "TATAC" and is complementary to a reference sequence "GTATA."

Genetic instability, as used herein, refers to the natural tendency of highly repetitive sequences to be lost through a process of reductive events generally involving

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the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (*i.e.*, the window size), and multiplying the result by 100 to yield the percentage of sequence identity. This "substantial identity", as used herein, denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence having at least 80 percent sequence identity, preferably at least 85 percent identity, often 90 to 95 percent sequence identity, and most commonly at least 99 percent sequence identity as compared to a reference sequence of a comparison window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison.

"Conservative amino acid substitutions" refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are : valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

The term "homologous" or "homeologous" means that one single-stranded nucleic acid nucleic acid sequence may hybridize to a complementary single-stranded nucleic acid sequence. The degree of hybridization may depend on a number of factors including the amount of identity between the sequences and the hybridization conditions such as temperature and salt concentrations as discussed later. Preferably the region of identity is greater than about 5 bp, more preferably the region of identity is greater than 10 bp.

The term "heterologous" means that one single-stranded nucleic acid sequence is unable to hybridize to another single-stranded nucleic acid sequence or its complement. Thus areas of heterology means that areas of polynucleotides or polynucleotides have



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The term "wild-type" means that the polynucleotide does not comprise any mutations. A "wild type" protein means that the protein will be active at a level of activity found in nature and will comprise the amino acid sequence found in nature.

In the polypeptide notation used herein, the left-hand direction is the amino terminal direction and the right-hand direction is the carboxy-terminal direction, in accordance with standard usage and convention. Similarly, unless specified otherwise, the left-hand end of single-stranded polynucleotide sequences is the 5' end; the left-hand direction of double-stranded polynucleotide sequences is referred to as the 5' direction. The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand having the same sequence as the RNA and which are 5' to the 5' end of the RNA transcript are referred to as "upstream sequences"; sequence regions on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the coding RNA transcript are referred to as "downstream sequences".

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As used herein, "substantially pure" means an object species is the predominant species present (*i.e.*, on a molar basis it is more abundant than any other individual macromolecular species in the composition), and preferably substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 to 90 percent of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species. Solvent species, small molecules (<500 Daltons), and elemental ion species are not considered macromolecular species.

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typical laboratory culture conditions are physiological conditions. Suitable *in vitro* reaction conditions for *in vitro* transcription cocktails are generally physiological conditions. In general, *in vitro* physiological conditions comprise 50-200 mM NaCl or KCl, pH 6.5-8.5, 20-45 °C and 0.001-10 mM divalent cation (*e.g.*, Mg⁺⁺, Ca⁺⁺); preferably about 150 mM NaCl or KCl, pH 7.2-7.6, 5 mM divalent cation, and often include 0.01-1.0 percent nonspecific protein (*e.g.*, BSA). A non-ionic detergent (Tween, NP-40, Triton X-100) can often be present, usually at about 0.001 to 2%, typically 0.05-0.2% (v/v). Particular aqueous conditions may be selected by the practitioner according to conventional methods. For general guidance, the following buffered aqueous conditions may be applicable: 10-250 mM NaCl, 5-50 mM Tris HCl, pH 5-8, with optional addition of divalent cation(s) and/or metal chelators and/or non-ionic detergents and/or membrane fractions and/or anti-foam agents and/or scintillants.

"Specific hybridization" is defined herein as the formation of hybrids between a first polynucleotide and a second polynucleotide (*e.g.*, a polynucleotide having a distinct but substantially identical sequence to the first polynucleotide), wherein substantially unrelated polynucleotide sequences do not form hybrids in the mixture.

As used herein, the term "single-chain antibody" refers to a polypeptide comprising a V_H domain and a V_L domain in polypeptide linkage, generally linked via a spacer peptide (*e.g.*, [Gly-Gly-Gly-Gly-Ser]_x), and which may comprise additional amino acid sequences at the amino- and/or carboxy- termini. For example, a single-chain antibody may comprise a tether segment for linking to the encoding polynucleotide. As an example, a scFv is a single-chain antibody. Single-chain antibodies are generally proteins consisting of one or more polypeptide segments of at least 10 contiguous amino substantially encoded by genes of the immunoglobulin superfamily (*e.g.*, see The Immunoglobulin Gene Superfamily, A.F. Williams and A.N. Barclay, in Immunoglobulin Genes, T. Honjo, F.W. Alt, and THE. Rabbits, eds., (1989) Academic press: San Diego, CA, pp. 361-368, which is incorporated herein by reference), most frequently encoded by a rodent, non-human primate, avian, porcine bovine, ovine, goat, or human heavy chain or light chain gene sequence. A functional single-chain antibody generally contains a sufficient portion

of an immunoglobulin superfamily gene product so as to retain the property of binding to a specific target molecule, typically a receptor or antigen (epitope).

As used herein, the term "complementarity-determining region" and "CDR" refer to the art-recognized term as exemplified by the Kabat and Chothia CDR definitions also generally known as supervariable regions or hypervariable loops (Chothia and Leks (1987) J. Mol. Biol. 196; 901; Clothia *et al.* (1989) Nature 342; 877; E.A. Kabat *et al.*, Sequences of Proteins of Immunological Interest (national Institutes of Health, Bethesda, MD) (1987); and Tramontano *et al.* (1990) J. Mol. Biolog. 215; 175). Variable region domains typically comprise the amino-terminal approximately 105-115 amino acids of a naturally-occurring immunoglobulin chain (*e.g.*, amino acids 1-110), although variable domains somewhat shorter or longer are also suitable for forming single-chain antibodies.

An immunoglobulin light or heavy chain variable region consists of a "framework" region interrupted by three hypervariable regions, also called CDR's. The extent of the framework region and CDR's have been precisely defined (see, "Sequences of Proteins of Immunological Interest," E. Kabat *et al.*, 4th Ed., U.S. Department of Health and human services, Bethesda, MD (1987)). The sequences of the framework regions of different light or heavy chains are relatively conserved within a specie. As used herein, a "human framework region" is a framework region that is substantially identical (about 85 or more, usually 90-95 or more) to the framework region of a naturally occurring human immunoglobulin. the framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDR's. The CDR's are primarily responsible for binding to an epitope of an antigen.

As used herein, the term "variable segment" refers to a portion of a nascent peptide which comprises a random, pseudorandom, or defined kernal sequence. A variable segment" refers to a portion of a nascent peptide which comprises a random pseudorandom, or defined kernal sequence. A variable segment can comprise both variant and invariant residue positions, and the degree of residue variation at a variant residue position may be limited: both options are selected at the discretion of the practitioner. Typically, variable segments are about 5 to 20 amino acid residues in length (*e.g.*, 8 to 10), although variable segments may be longer and may comprise antibody portions or receptor

gble

Abstract. We study the asymptotic behavior of the eigenvalues of the Dirac operator $D_{\mathbb{H}^n}$ on the hyperbolic space \mathbb{H}^n with a constant magnetic field. We show that the eigenvalues are asymptotically distributed according to a certain probability measure on the real line. This measure is related to the spectral measure of the Dirac operator on the real line.

As used herein "random peptide library" refers to a set of polynucleotide sequences that encodes a set of random peptides, and to the set of random peptides encoded by those polynucleotide sequences, as well as the fusion proteins contain those random peptides.

As used herein, the term "defined sequence framework" refers to a set of defined sequences that are selected on a non-random basis, generally on the basis of experimental data or structural data; for example, a defined sequence framework may comprise a set of amino acid sequences that are predicted to form a β -sheet structure or may comprise a leucine zipper heptad repeat motif, a zinc-finger domain, among other variations. A "defined sequence kernel" is a set of sequences which encompass a limited scope of variability. Whereas (1) a completely random 10-mer sequence of the 20 conventional amino acids can be any of $(20)^{10}$ sequences, and (2) a pseudorandom 10-mer sequence of the 20 conventional amino acids can be any of $(20)^{10}$ sequences but will exhibit a bias for certain residues at certain positions and/or overall, (3) a defined sequence kernel is a subset of sequences if each residue position was allowed to be any of the allowable 20 conventional amino acids (and/or allowable unconventional amino/imino acids). A defined sequence kernel generally comprises variant and invariant residue positions and/or comprises variant residue positions which can comprise a residue selected from a defined subset of amino acid residues), and the like, either segmentally or over the entire length of the individual selected library member sequence. Defined sequence kernels can refer to

[illegible]

As used herein, "receptor" refers to a molecule that has an affinity for a given ligand. Receptors can be naturally occurring or synthetic molecules. Receptors can be employed in an unaltered state or as aggregates with other species. Receptors can be attached, covalently or non-covalently, to a binding member, either directly or via a specific binding substance. Examples of receptors include, but are not limited to, antibodies, including monoclonal antibodies and antisera reactive with specific antigenic determinants (such as on viruses, cells, or other materials), cell membrane receptors, complex carbohydrates and glycoproteins, enzymes, and hormone receptors.

As used herein "ligand" refers to a molecule, such as a random peptide or variable segment sequence, that is recognized by a particular receptor. As one of skill in the art will recognize, a molecule (or macromolecular complex) can be both a receptor and a ligand. In general, the binding partner having a smaller molecular weight is referred to as the ligand and the binding partner having a greater molecular weight is referred to as a receptor.

As used herein, "linker" or "spacer" refers to a molecule or group of molecules that connects two molecules, such as a DNA binding protein and a random peptide, and serves to place the two molecules in a preferred configuration, *e.g.*, so that the random peptide can bind to a receptor with minimal steric hindrance from the DNA binding protein.

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1. *Chlorophyll a* (Chl *a*)
 2. *Chlorophyll b* (Chl *b*)
 3. *Chlorophyll c* (Chl *c*)
 4. *Chlorophyll d* (Chl *d*)
 5. *Chlorophyll e* (Chl *e*)
 6. *Chlorophyll f* (Chl *f*)
 7. *Chlorophyll g* (Chl *g*)
 8. *Chlorophyll h* (Chl *h*)
 9. *Chlorophyll i* (Chl *i*)
 10. *Chlorophyll j* (Chl *j*)
 11. *Chlorophyll k* (Chl *k*)
 12. *Chlorophyll l* (Chl *l*)
 13. *Chlorophyll m* (Chl *m*)
 14. *Chlorophyll n* (Chl *n*)
 15. *Chlorophyll o* (Chl *o*)
 16. *Chlorophyll p* (Chl *p*)
 17. *Chlorophyll q* (Chl *q*)
 18. *Chlorophyll r* (Chl *r*)
 19. *Chlorophyll s* (Chl *s*)
 20. *Chlorophyll t* (Chl *t*)
 21. *Chlorophyll u* (Chl *u*)
 22. *Chlorophyll v* (Chl *v*)
 23. *Chlorophyll w* (Chl *w*)
 24. *Chlorophyll x* (Chl *x*)
 25. *Chlorophyll y* (Chl *y*)
 26. *Chlorophyll z* (Chl *z*)
 27. *Chlorophyll aa* (Chl *aa*)
 28. *Chlorophyll ab* (Chl *ab*)
 29. *Chlorophyll ac* (Chl *ac*)
 30. *Chlorophyll ad* (Chl *ad*)
 31. *Chlorophyll ae* (Chl *ae*)
 32. *Chlorophyll af* (Chl *af*)
 33. *Chlorophyll ag* (Chl *ag*)
 34. *Chlorophyll ah* (Chl *ah*)
 35. *Chlorophyll ai* (Chl *ai*)
 36. *Chlorophyll aj* (Chl *aj*)
 37. *Chlorophyll ak* (Chl *ak*)
 38. *Chlorophyll al* (Chl *al*)
 39. *Chlorophyll am* (Chl *am*)
 40. *Chlorophyll an* (Chl *an*)
 41. *Chlorophyll ao* (Chl *ao*)
 42. *Chlorophyll ap* (Chl *ap*)
 43. *Chlorophyll aq* (Chl *aq*)
 44. *Chlorophyll ar* (Chl *ar*)
 45. *Chlorophyll as* (Chl *as*)
 46. *Chlorophyll at* (Chl *at*)
 47. *Chlorophyll au* (Chl *au*)
 48. *Chlorophyll av* (Chl *av*)
 49. *Chlorophyll aw* (Chl *aw*)
 50. *Chlorophyll ax* (Chl *ax*)
 51. *Chlorophyll ay* (Chl *ay*)
 52. *Chlorophyll az* (Chl *az*)
 53. *Chlorophyll aza* (Chl *aza*)
 54. *Chlorophyll abz* (Chl *abz*)
 55. *Chlorophyll acz* (Chl *acz*)
 56. *Chlorophyll adz* (Chl *adz*)
 57. *Chlorophyll aez* (Chl *aez*)
 58. *Chlorophyll afz* (Chl *afz*)
 59. *Chlorophyll agz* (Chl *agz*)
 60. *Chlorophyll ahz* (Chl *ahz*)
 61. *Chlorophyll aiz* (Chl *aiz*)
 62. *Chlorophyll ajz* (Chl *ajz*)
 63. *Chlorophyll akz* (Chl *akz*)
 64. *Chlorophyll alz* (Chl *alz*)
 65. *Chlorophyll amz* (Chl *amz*)
 66. *Chlorophyll anz* (Chl *anz*)
 67. *Chlorophyll aoz* (Chl *aoz*)
 68. *Chlorophyll apz* (Chl *apz*)
 69. *Chlorophyll aqz* (Chl *aqz*)
 70. *Chlorophyll arz* (Chl *arz*)
 71. *Chlorophyll asz* (Chl *asz*)
 72. *Chlorophyll atz* (Chl *atz*)
 73. *Chlorophyll auz* (Chl *auz*)
 74. *Chlorophyll avz* (Chl *avz*)
 75. *Chlorophyll awz* (Chl *awz*)
 76. *Chlorophyll axz* (Chl *axz*)
 77. *Chlorophyll ayz* (Chl *ayz*)
 78. *Chlorophyll azz* (Chl *azz*)
 79. *Chlorophyll azaa* (Chl *aza*)
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 85. *Chlorophyll agz* (Chl *agz*)
 86. *Chlorophyll ahz* (Chl *ahz*)
 87. *Chlorophyll aiz* (Chl *aiz*)
 88. *Chlorophyll ajz* (Chl *ajz*)
 89. *Chlorophyll akz* (Chl *akz*)
 90. *Chlorophyll alz* (Chl *alz*)
 91. *Chlorophyll amz* (Chl *amz*)
 92. *Chlorophyll anz* (Chl *anz*)
 93. *Chlorophyll aoz* (Chl *aoz*)
 94. *Chlorophyll apz* (Chl *apz*)
 95. *Chlorophyll aqz* (Chl *aqz*)
 96. *Chlorophyll arz* (Chl *arz*)
 97. *Chlorophyll asz* (Chl *asz*)
 98. *Chlorophyll atz* (Chl *atz*)
 99. *Chlorophyll auz* (Chl *auz*)
 100. *Chlorophyll avz* (Chl *avz*)
 101. *Chlorophyll awz* (Chl *awz*)
 102. *Chlorophyll axz* (Chl *axz*)
 103. *Chlorophyll ayz* (Chl *ayz*)
 104. *Chlorophyll azz* (Chl *azz*)
 105. *Chlorophyll azaa* (Chl *aza*)
 106. *Chlorophyll abz* (Chl *abz*)
 107. *Chlorophyll acz* (Chl *acz*)
 108. *Chlorophyll adz* (Chl *adz*)
 109. *Chlorophyll aez* (Chl *aez*)
 110. *Chlorophyll afz* (Chl *afz*)
 111. *Chlorophyll agz* (Chl *agz*)
 112. *Chlorophyll ahz* (Chl *ahz*)
 113. *Chlorophyll aiz* (Chl *aiz*)
 114. *Chlorophyll ajz* (Chl *ajz*)
 115. *Chlorophyll akz* (Chl *akz*)
 116. *Chlorophyll alz* (Chl *alz*)
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 118. *Chlorophyll anz* (Chl *anz*)
 119. *Chlorophyll aoz* (Chl *aoz*)
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 121. *Chlorophyll aqz* (Chl *aqz*)
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 132. *Chlorophyll abz* (Chl *abz*)
 133.

In contrast to cassette mutagenesis, only shuffling and error-prone PCR allow one to mutate a pool of sequences blindly (without sequence information other than primers).

The advantage of the mutagenic shuffling of this invention over error-prone PCR alone for repeated selection can best be explained with an example from antibody engineering.

This method differs from error-prone PCR, in that it is an inverse chain reaction. In error-prone PCR, the number of polymerase start sites and the number of molecules grows exponentially. However, the sequence of the polymerase start sites and the sequence of the molecules remains essentially the same. In contrast, in nucleic acid reassembly or shuffling of random polynucleotides the number of start sites and the number (but not size) of the random polynucleotides decreases over time. For polynucleotides derived from whole plasmids the theoretical endpoint is a single, large concatemeric molecule.

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The double-stranded polynucleotide template and any added double-or single-stranded polynucleotides are subjected to sexual PCR which includes slowing or halting to provide a mixture of from about 5 bp to 5 kb or more. Preferably the size of the random polynucleotides is from about 10 bp to 1000 bp, more preferably the size of the polynucleotides is from about 20 bp to 500 bp.

Alternatively, it is also contemplated that double-stranded nucleic acid having multiple nicks may be used in the methods of this invention. A nick is a break in one strand of the double-stranded nucleic acid. The distance between such nicks is preferably 5 bp to 5 kb, more preferably between 10 bp to 1000 bp. This can provide areas of self-priming to produce shorter or smaller polynucleotides to be included with the polynucleotides resulting from random primers, for example.

The number of different specific polynucleotides in the mixture will be at least about 100, preferably at least about 500, and more preferably at least about 1000.

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It is also contemplated that populations of double-stranded randomly broken polynucleotides may be mixed or combined at this step with the polynucleotides from the sexual PCR process and optionally subjected to one or more additional sexual PCR cycles.

Where a mixture of different but related template polynucleotides is desired, populations of polynucleotides from each of the templates may be combined at a ratio of less than about 1:100, more preferably the ratio is less than about 1:40. For example, a backcross of the wild-type polynucleotide with a population of mutated polynucleotide may be desired to eliminate neutral mutations (*e.g.*, mutations yielding an insubstantial alteration in the phenotypic property being selected for). In such an example, the ratio of randomly provided wild-type polynucleotides which may be added to the randomly provided sexual PCR cycle hybrid polynucleotides is approximately 1:1 to about 100:1, and more preferably from 1:1 to 40:1.

The mixed population of random polynucleotides are denatured to form single-stranded polynucleotides and then re-annealed. Only those single-stranded polynucleotides having regions of homology with other single-stranded polynucleotides will re-anneal.

[illegible]

8.16.5.3.6 The Resulting Nucleic Acid

This larger polynucleotides may contain a number of copies of a polynucleotide having the same size as the template polynucleotide in tandem. This concatemeric polynucleotide is then denatured into single copies of the template polynucleotide. The result will be a population of polynucleotides of approximately the same size as the template polynucleotide. The population will be a mixed population where single or double-stranded polynucleotides having an area of identity and an area of heterology have been added to the template polynucleotide prior to shuffling.

It is contemplated that the single polynucleotides may be obtained from the larger concatemeric polynucleotide by amplification of the single polynucleotide prior to cloning by a variety of methods including PCR (U.S. Patent No. 4,683,195 and 4,683,202), rather than by digestion of the concatemer.

The vector used for cloning is not critical provided that it will accept a polynucleotide of the desired size. If expression of the particular polynucleotide is

desired, the cloning vehicle should further comprise transcription and translation signals next to the site of insertion of the polynucleotide to allow expression of the polynucleotide in the host cell. Preferred vectors include the pUC series and the pBR series of plasmids.

8.16.5.3.8 The Resulting Bacterial Population

The resulting bacterial population will include a number of recombinant polynucleotides having random mutations. This mixed population may be tested to identify the desired recombinant polynucleotides. The method of selection will depend on the polynucleotide desired.

For example, if a polynucleotide which encodes a protein with increased binding efficiency to a ligand is desired, the proteins expressed by each of the portions of the polynucleotides in the population or library may be tested for their ability to bind to the ligand by methods known in the art (*i.e.* panning, affinity chromatography). If a polynucleotide which encodes for a protein with increased drug resistance is desired, the proteins expressed by each of the polynucleotides in the population or library may be tested for their ability to confer drug resistance to the host organism. One skilled in the art, given knowledge of the desired protein, could readily test the population to identify polynucleotides which confer the desired properties onto the protein.

It is contemplated that one skilled in the art could use a phage display system in which fragments of the protein are expressed as fusion proteins on the phage surface (Pharmacia, Milwaukee WI). The recombinant DNA molecules are cloned into the phage DNA at a site which results in the transcription of a fusion protein a portion of which is encoded by the recombinant DNA molecule. The phage containing the recombinant nucleic acid molecule undergoes replication and transcription in the cell. The leader sequence of the fusion protein directs the transport of the fusion protein to the tip of the phage particle. Thus the fusion protein which is partially encoded by the recombinant DNA molecule is displayed on the phage particle for detection and selection by the methods described above.

8.16.5.3.9 Cycles of Nucleic Acid Shuffling

It is further contemplated that a number of cycles of nucleic acid shuffling may be conducted with polynucleotides from a sub-population of the first population, which sub-population contains DNA encoding the desired recombinant protein. In this manner, proteins with even higher binding affinities or enzymatic activity could be achieved.

It is also contemplated that a number of cycles of nucleic acid shuffling may be conducted with a mixture of wild-type polynucleotides and a sub-population of nucleic acid from the first or subsequent rounds of nucleic acid shuffling in order to remove any silent mutations from the sub-population.

8.16.5.3.10 The Starting Nucleic Acid

Any source of nucleic acid, in purified form can be utilized as the starting nucleic acid. Thus the process may employ DNA or RNA including messenger RNA, which DNA or RNA may be single or double stranded. In addition, a DNA-RNA hybrid which contains one strand of each may be utilized. The nucleic acid sequence may be of various lengths depending on the size of the nucleic acid sequence to be mutated. Preferably the specific nucleic acid sequence is from 50 to 50000 base pairs. It is contemplated that entire vectors containing the nucleic acid encoding the protein of interest may be used in the methods of this invention.

The nucleic acid may be obtained from any source, for example, from plasmids such a pBR322, from cloned DNA or RNA or from natural DNA or RNA from any source including bacteria, yeast, viruses and higher organisms such as plants or animals. DNA or RNA may be extracted from blood or tissue material. The template polynucleotide may be obtained by amplification using the polynucleotide chain reaction (PCR) (U.S. Patent no. 4,683,202 and 4,683,195). Alternatively, the polynucleotide may be present in a vector present in a cell and sufficient nucleic acid may be obtained by culturing the cell and extracting the nucleic acid from the cell by methods known in the art.

Any specific nucleic acid sequence can be used to produce the population of hybrids by the present process. It is only necessary that a small population of hybrid

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The \mathcal{H}^1 norm of the function f is defined as

$$\|f\|_{\mathcal{H}^1} = \left(\int_{\mathbb{R}^d} |\nabla f|^2 dx \right)^{1/2}.$$
 The \mathcal{H}^1 norm of the function f is defined as

$$\|f\|_{\mathcal{H}^1} = \left(\int_{\mathbb{R}^d} |\nabla f|^2 dx \right)^{1/2}.$$

The initial small population of the specific nucleic acid sequences having mutations may be created by a number of different methods. Mutations may be created by error-prone PCR. Error-prone PCR uses low-fidelity polymerization conditions to introduce a low level of point mutations randomly over a long sequence. Alternatively, mutations can be introduced into the template polynucleotide by oligonucleotide-directed mutagenesis. In oligonucleotide-directed mutagenesis, a short sequence of the polynucleotide is removed from the polynucleotide using restriction enzyme digestion and is replaced with a synthetic polynucleotide in which various bases have been altered from the original sequence. The polynucleotide sequence can also be altered by chemical mutagenesis. Chemical mutagens include, for example, sodium bisulfite, nitrous acid, hydroxylamine, hydrazine or formic acid. other agents which are analogues of nucleotide precursors include nitrosoguanidine, 5-bromouracil, 2-aminopurine, or acridine. Generally, these agents are added to the PCR reaction in place of the nucleotide precursor thereby mutating the sequence. Intercalating agents such as proflavine, acriflavine, quinacrine and the like can also be used. Random mutagenesis of the polynucleotide sequence can also be achieved by irradiation with X-rays or ultraviolet light. Generally, plasmid polynucleotides so mutagenized are introduced into *E. coli* and propagated as a pool or library of hybrid plasmids.

Alternatively the small mixed population of specific nucleic acids may be found in nature in that they may consist of different alleles of the same gene or the same gene from different related species (*i.e.*, cognate genes). Alternatively, they may be related DNA sequences found within one species, for example, the immunoglobulin genes.

Once the mixed population of the specific nucleic acid sequences is generated, the polynucleotides can be used directly or inserted into an appropriate cloning vector, using techniques well-known in the art.

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Shuffling requires the presence of homologous regions separating regions of diversity. Scaffold-like protein structures may be particularly suitable for shuffling. The conserved scaffold determines the overall folding by self-association, while displaying relatively unrestricted loops that mediate the specific binding. Examples of such scaffolds are the immunoglobulin beta-barrel, and the four-helix bundle which are well-known in the art. This shuffling can be used to create scaffold-like proteins with various combinations of mutated sequences for binding.

The equivalents of some standard genetic matings may also be performed by shuffling *in vitro*. For example, a "molecular backcross" can be performed by repeatedly mixing the hybrid's nucleic acid with the wild-type nucleic acid while selecting for the mutations of interest. As in traditional breeding, this approach can be used to combine phenotypes from different sources into a background of choice. It is useful, for example, for the removal of neutral mutations that affect unselected characteristics (*i.e.* immunogenicity). Thus it can be useful to determine which mutations in a protein are involved in the enhanced biological activity and which are not, an advantage which cannot be achieved by error-prone mutagenesis or cassette mutagenesis methods.

Large, functional genes can be assembled correctly from a mixture of small random polynucleotides. This reaction may be of use for the reassembly of genes from the highly fragmented DNA of fossils. In addition random nucleic acid fragments from fossils may be combined with polynucleotides from similar genes from related species.

8.16.5.4.1 *In Vitro* Amplification of a Genome

It is also contemplated that the method of this invention can be used for the *in vitro* amplification of a whole genome from a single cell as is needed for a variety of research and diagnostic applications. DNA amplification by PCR is in practice limited to a length of about 40 kb. Amplification of a whole genome such as that of *E. coli* (5,000 kb) by PCR would require about 250 primers yielding 125 forty kb polynucleotides. This approach is not practical due to the unavailability of sufficient sequence data. On the other hand, random production of polynucleotides of the genome with sexual PCR cycles, followed by gel purification of small polynucleotides will provide a multitude of possible primers. Use of this mix of random small polynucleotides as primers in a PCR reaction alone or with the whole genome as the template should result in an inverse chain reaction with the theoretical endpoint of a single concatemer containing many copies of the genome.

100 fold amplification in the copy number and an average polynucleotide size of greater than 50 kb may be obtained when only random polynucleotides are used. It is thought that the larger concatemer is generated by overlap of many smaller polynucleotides. The quality of specific PCR products obtained using synthetic primers will be indistinguishable from the product obtained from unamplified DNA. It is expected that this approach will be useful for the mapping of genomes.

The polynucleotide to be shuffled can be produced as random or non-random polynucleotides, at the discretion of the practitioner.

It is also contemplated that in the second or subsequent recombination cycle, a backcross can be performed. A molecular backcross can be performed by mixing the desired specific nucleic acid sequences with a large number of the wild-type sequence, such that at least one wild-type nucleic acid sequence and a mutated nucleic acid sequence are present in the same host cell after transformation. Recombination with the wild-type specific nucleic acid sequence will eliminate those neutral mutations that may affect unselected characteristics such as immunogenicity but not the selected characteristics.

8.16.5.5.5 Generation of a Subset of the Specific Nucleic Acid Sequences

In another embodiment of this invention, it is contemplated that during the first round a subset of the specific nucleic acid sequences can be generated as smaller polynucleotides by slowing or halting their PCR amplification prior to introduction into the host cell. The size of the polynucleotides must be large enough to contain some regions of identity with the other sequences so as to homologously recombine with the other sequences. The size of the polynucleotides will range from 0.03 kb to 100 kb more preferably from 0.2 kb to 10 kb. It is also contemplated that in subsequent rounds, all of the specific nucleic acid sequences other than the sequences selected from the previous round may be utilized to generate PCR polynucleotides prior to introduction into the host cells.

The shorter polynucleotide sequences can be single-stranded or double-stranded. If the sequences were originally single-stranded and have become double-stranded they can be denatured with heat, chemicals or enzymes prior to insertion into the host cell. The reaction conditions suitable for separating the strands of nucleic acid are well known in the art.

The steps of this process can be repeated indefinitely, being limited only by the number of possible hybrids which can be achieved. After a certain number of cycles, all possible hybrids will have been achieved and further cycles are redundant.

In an embodiment the same mutated template nucleic acid is repeatedly recombined and the resulting recombinants selected for the desired characteristic.

1. The first part of the document is a list of names and their corresponding addresses. The names are listed in a column on the left, and the addresses are listed in a column on the right. The names are: John Doe, Jane Smith, and Bob Johnson. The addresses are: 123 Main St, 456 Elm St, and 789 Oak St.

It has been shown that by this method nucleic acid sequences having enhanced desired properties can be selected.

In an alternate embodiment, the first generation of hybrids are retained in the cells and the parental mutated sequences are added again to the cells. Accordingly, the first cycle of Embodiment I is conducted as described above. However, after the daughter nucleic acid sequences are identified, the host cells containing these sequences are retained.

This cycle can be repeated a number of times until the nucleic acid or peptide having the desired characteristics is obtained. It is contemplated that in subsequent cycles, the population of mutated sequences which are added to the preferred hybrids may come from the parental hybrids or any subsequent generation.

8.16.5.5.10 "Molecular" Backcross to Eliminate Any Neutral Mutations

In an alternative embodiment, the invention provides a method of conducting a "molecular" backcross of the obtained recombinant specific nucleic acid in order to eliminate any neutral mutations. Neutral mutations are those mutations which do not confer onto the nucleic acid or peptide the desired properties. Such mutations may however confer on the nucleic acid or peptide undesirable characteristics. Accordingly, it is desirable to eliminate such neutral mutations. The method of this invention provide a means of doing so.

In this embodiment, after the hybrid nucleic acid, having the desired characteristics, is obtained by the methods of the embodiments, the nucleic acid, the vector having the nucleic acid or the host cell containing the vector and nucleic acid is isolated.

The nucleic acid or vector is then introduced into the host cell with a large excess of the wild-type nucleic acid. The nucleic acid of the hybrid and the nucleic acid of the wild-type sequence are allowed to recombine. The resulting recombinants are placed under the same selection as the hybrid nucleic acid. Only those recombinants which retained the desired characteristics will be selected. Any silent mutations which do not provide the desired characteristics will be lost through recombination with the wild-type DNA. This cycle can be repeated a number of times until all of the silent mutations are eliminated.

Thus the methods of this invention can be used in a molecular backcross to eliminate unnecessary or silent mutations.

8.16.5.6 Utility

The *in vivo* recombination method of this invention can be performed blindly on a pool of unknown hybrids or alleles of a specific polynucleotide or sequence. However, it is not necessary to know the actual DNA or RNA sequence of the specific polynucleotide.

The approach of using recombination within a mixed population of genes can be useful for the generation of any useful proteins, for example, interleukin I, antibodies, tPA and growth hormone. This approach may be used to generate proteins having altered

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portion with DNA binding activity and a second polypeptide portion having the library member unique peptide sequence; such methods are suitable for use in cell-free *in vitro* selection formats, among others.

8.16.5.7.2 The Displayed Peptide Sequences

The displayed peptide sequences can be of varying lengths, typically from 3-5000 amino acids long or longer, frequently from 5-100 amino acids long, and often from about 8-15 amino acids long. A library can comprise library members having varying lengths of displayed peptide sequence, or may comprise library members having a fixed length of displayed peptide sequence. Portions or all of the displayed peptide sequence(s) can be random, pseudorandom, defined set kernel, fixed, or the like. The present display methods include methods for *in vitro* and *in vivo* display of single-chain antibodies, such as nascent scFv on polysomes or scfv displayed on phage, which enable large-scale screening of scfv libraries having broad diversity of variable region sequences and binding specificities.

8.16.5.7.3 Sequence Framework Peptide Libraries

The present invention also provides random, pseudorandom, and defined sequence framework peptide libraries and methods for generating and screening those libraries to identify useful compounds (*e.g.*, peptides, including single-chain antibodies) that bind to receptor molecules or epitopes of interest or gene products that modify peptides or RNA in a desired fashion. The random, pseudorandom, and defined sequence framework peptides are produced from libraries of peptide library members that comprise displayed peptides or displayed single-chain antibodies attached to a polynucleotide template from which the displayed peptide was synthesized. The mode of attachment may vary according to the specific embodiment of the invention selected, and can include encapsulation in a phage particle or incorporation in a cell.

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8.16.5.7.4 Selecting for the Desired Peptide Using Affinity Enrichment

A method of affinity enrichment allows a very large library of peptides and single-chain antibodies to be screened and the polynucleotide sequence encoding the desired peptide(s) or single-chain antibodies to be selected. The polynucleotide can then be isolated and shuffled to recombine combinatorially the amino acid sequence of the selected peptide(s) (or predetermined portions thereof) or single-chain antibodies (or just VHI, VLI or CDR portions thereof). Using these methods, one can identify a peptide or single-chain antibody as having a desired binding affinity for a molecule and can exploit the process of shuffling to converge rapidly to a desired high-affinity peptide or scfv. The peptide or antibody can then be synthesized in bulk by conventional means for any suitable use (*e.g.*, as a therapeutic or diagnostic agent).

A significant advantage of the present invention is that no prior information regarding an expected ligand structure is required to isolate peptide ligands or antibodies of interest. The peptide identified can have biological activity, which is meant to include at least specific binding affinity for a selected receptor molecule and, in some instances, will further include the ability to block the binding of other compounds, to stimulate or inhibit metabolic pathways, to act as a signal or messenger, to stimulate or inhibit cellular activity, and the like.

8.16.5.7.5 Shuffling Sequences Selected by Affinity Screening

The present invention also provides a method for shuffling a pool of polynucleotide sequences selected by affinity screening a library of polysomes displaying nascent peptides (including single-chain antibodies) for library members which bind to a predetermined receptor (*e.g.*, a mammalian proteinaceous receptor such as, for example, a peptidergic hormone receptor, a cell surface receptor, an intracellular protein which binds to other protein(s) to form intracellular protein complexes such as hetero-dimers and the like) or epitope (*e.g.*, an immobilized protein, glycoprotein, oligosaccharide, and the like).

Polynucleotide sequences selected in a first selection round (typically by affinity selection for binding to a receptor (*e.g.*, a ligand)) by any of these methods are pooled and

8.16.5.8.2 Prokaryotic Expression Systems

In order to overcome many of the limitations in producing and identifying high-affinity immunoglobulins through antigen-stimulated β cell development (*i.e.*, immunization), various prokaryotic expression systems have been developed that can be manipulated to produce combinatorial antibody libraries which may be screened for high-affinity antibodies to specific antigens. Recent advances in the expression of antibodies in *Escherichia coli* and bacteriophage systems (*see*, "Alternative Peptide Display Methods", *infra*) have raised the possibility that virtually any specificity can be obtained by either cloning antibody genes from characterized hybridomas or by *de novo* selection using antibody gene libraries (*e.g.*, from Ig CDNA).

Combinatorial libraries of antibodies have been generated in bacteriophage lambda expression systems which may be screened as bacteriophage plaques or as colonies of lysogens (Huse *et al.* (1989) Science 246: 1275; Caton and Koprowski (1990) Proc. Natl. Acad. Sci. (U.S.A.) 87: 6450; Mullinax *et al.* (1990) Proc. Natl. Acad. Sci. (U.S.A.) 87: 8095; Persson *et al.* (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88: 2432). Various embodiments of bacteriophage antibody display libraries and lambda phage expression libraries have been described (Kang *et al.* (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88: 4363; Clackson *et al.* (1991) Nature 352: 624; McCafferty *et al.* (1990) Nature 348: 552; Burton *et al.* (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88: 10134; Hoogenboom *et al.* (1991) Nucleic Acids Res. 19: 4133; Chang *et al.* (1991) J. Immunol. 147: 3610; Breitling *et al.* (1991) Gene 104: 147; Marks *et al.* (1991) J. Mol. Biol. 222@: 581; Barbas *et al.* (1992) Proc. Natl. Acad. Sci. (U.S.A.) 89: 4457; Hawkins and Winter (1992) J. Immunol. 22: 867; Marks *et al.* (1992) Biotechnology 10: 779; Marks *et al.* (1992) J. Biol. Chem. 267: 16007; Lowman *et al.* (1991) Biochemistry 30: 10832; Lerner *et al.* (1992) Science. 258: 1313, incorporated herein by reference). Typically, a bacteriophage antibody display library is screened with a receptor (*e.g.*, polypeptide, carbohydrate, glycoprotein, nucleic acid) that is immobilized (*e.g.*, by covalent linkage to a chromatography resin to enrich for reactive phage by affinity chromatography) and/or labeled (*e.g.*, to screen plaque or colony lifts).

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Beginning in 1988, single-chain analogues of Fv fragments and their fusion proteins have been reliably generated by antibody engineering methods. The first step generally involves obtaining the genes encoding VH and VL domains with desired binding properties; these V genes may be isolated from a specific hybridoma cell line, selected from a combinatorial V-gene library, or made by V gene synthesis. The single-chain Fv is formed by connecting the component V genes with an oligonucleotide that encodes an appropriately designed linker peptide, such as (Gly-Gly-Gly-Gly-Ser)₃ or equivalent linker peptide(s). The linker bridges the C-terminus of the first V region and N-terminus of the second, ordered as either VH-linker-VL or VL-linker-VH. In principle, the scFv binding site can faithfully replicate both the affinity and specificity of its parent antibody combining site.

The linked polynucleotide of a library member provides the basis for replication of the library member after a screening or selection procedure, and also provides the basis for the determination, by nucleotide sequencing, of the identity of the displayed peptide sequence or VH and VL amino acid sequence. The displayed peptide (s) or single-chain

antibody (e. g., scfv) and/or its VH and VL domains or their CDRs can be cloned and expressed in a suitable expression system. often polynucleotides encoding the isolated VH and VL domains will be ligated to polynucleotides encoding constant regions (CH and CL) to form polynucleotides encoding complete antibodies (e.g., chimeric or fully-human), antibody fragments, and the like. Often polynucleotides encoding the isolated CDRs will be grafted into polynucleotides encoding a suitable variable region framework (and optionally constant regions) to form polynucleotides encoding complete antibodies (e.g., humanized or fully-human), antibody fragments, and the like. Antibodies can be used to isolate preparative quantities of the antigen by immunoaffinity chromatography. Various other uses of such antibodies are to diagnose and/or stage disease (e.g., neoplasia) and for therapeutic application to treat disease, such as for example: neoplasia, autoimmune disease, AIDS, cardiovascular disease, infections, and the like.

8.16.5.8.4 Increasing the Combinatorial Diversity of a SCFV Library

Various methods have been reported for increasing the combinatorial diversity of a scfv library to broaden the repertoire of binding species (idiotype spectrum) The use of PCR has permitted the variable regions to be rapidly cloned either from a specific hybridoma source or as a gene library from non-immunized cells, affording combinatorial diversity in the assortment of VH and VL cassettes which can be combined. Furthermore, the VH and VL cassettes can themselves be diversified, such as by random, pseudorandom, or directed mutagenesis. Typically, VH and VL cassettes are diversified in or near the complementarity-determining regions (CDRS), often the third CDR, CDR3. Enzymatic inverse PCR mutagenesis has been shown to be a simple and reliable method for constructing relatively large libraries of scfv site-directed hybrids (Stemmer *et al.* (1993) Biotechniques 14: 256), as has error-prone PCR and chemical mutagenesis (Deng *et al.* (1994) J. Biol. Chem. 269: 953 3). Riechmann *et al.* (1993) Biochemistry 32: 8848 showed semi-rational design of an antibody scfv fragment using site-directed randomization by degenerate oligonucleotide PCR and subsequent phage display of the resultant scfv hybrids. Barbas *et al.* (1992) on.cit. attempted to circumvent the problem of

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8.16.5.8.5 Use of *in vitro* and *in vivo* Shuffling Methods to Recombine CDRs

If it were possible to generate scfv libraries having broader antibody diversity and overcoming many of the limitations of conventional CDR mutagenesis and randomization methods which can cover only a very tiny fraction of the potential sequence combinations, the number and quality of scfv antibodies suitable for therapeutic and diagnostic use could be vastly improved. To address this, the *in vitro* and *in vivo* shuffling methods of the invention are used to recombine CDRs which have been obtained (typically via PCR amplification or cloning) from nucleic acids obtained from selected displayed antibodies. Such displayed antibodies can be displayed on cells, on bacteriophage particles, on polysomes, or any suitable antibody display system wherein the antibody is associated with its encoding nucleic acid(s). In a variation, the CDRs are initially obtained from MRNA (or cDNA) from antibody-producing cells (*e.g.*, plasma cells/splenocytes from an immunized wild-type mouse, a human, or a transgenic mouse capable of making a human antibody as in W092/03918, W093/12227, and W094/25585), including hybridomas derived therefrom.

Polynucleotide sequences selected in a first selection round (typically by affinity selection for displayed antibody binding to an antigen (*e.g.*, a ligand) by any of these methods are pooled and the pool(s) is/are shuffled by *in vitro* and/or *in vivo* recombination, especially shuffling of CDRs (typically shuffling heavy chain CDRs with other heavy chain CDRs and light chain CDRs with other light chain CDRs) to produce a shuffled pool comprising a population of recombined selected polynucleotide sequences. The recombined selected polynucleotide sequences are expressed in a selection format as a displayed antibody and subjected to at least one subsequent selection round. The polynucleotide sequences selected in the subsequent selection round(s) can be used directly, sequenced, and/or subjected to one or more additional rounds of shuffling and subsequent selection until an antibody of the desired binding affinity is obtained. Selected sequences can also be back-crossed with polynucleotide sequences encoding neutral antibody framework sequences (*i.e.*, having insubstantial functional effect on antigen binding), such as for example by back-crossing with a human variable region framework to produce human-like sequence antibodies. Generally, during back-crossing subsequent selection is applied to retain the property of binding to the predetermined antigen.

and incubated with a polysome-display scfv library under suitable binding conditions. The collection of beads, comprising multiple epitope species, can then be used to isolate, by affinity selection, scfv library members. Generally, subsequent affinity screening rounds can include the same mixture of beads, subsets thereof, or beads containing only one or two individual epitope species. This approach affords efficient screening, and is compatible with laboratory automation, batch processing, and high throughput screening methods.

8.16.5.8.8 Techniques Used to Diversify a Peptide Library or Single-Chain Antibody Library

A variety of techniques can be used in the present invention to diversify a peptide library or single-chain antibody library, or to diversify, prior to or concomitant with shuffling, around variable segment peptides found in early rounds of panning to have sufficient binding activity to the predetermined macromolecule or epitope. In one approach, the positive selected peptide/polynucleotide complexes (those identified in an early round of affinity enrichment) are sequenced to determine the identity of the active peptides. Oligonucleotides are then synthesized based on these active peptide sequences, employing a low level of all bases incorporated at each step to produce slight variations of the primary oligonucleotide sequences. This mixture of (slightly) degenerate oligonucleotides is then cloned into the variable segment sequences at the appropriate locations. This method produces systematic, controlled variations of the starting peptide sequences, which can then be shuffled. It requires, however, that individual positive nascent peptide/polynucleotide complexes be sequenced before mutagenesis, and thus is useful for expanding the diversity of small numbers of recovered complexes and selecting variants having higher binding affinity and/or higher binding specificity. In a variation, mutagenic PCR amplification of positive selected peptide/polynucleotide complexes (especially of the variable region sequences, the amplification products of which are shuffled *in vitro* and/or *in vivo* and one or more additional rounds of screening is done prior to sequencing. The same general approach can be employed with single-chain antibodies in order to expand the diversity and enhance the binding affinity/specificity,

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sequences have carboxy-terminal amino acids which are either Tyr, Val, Phe, or Asp, then the pool(s) of synthetic CDR oligonucleotide sequences are designed to allow the carboxy-terminal CDR residue to be any of these amino acids. In some embodiments, residues other than those which naturally-occur at a residue position in the collection of CDR sequences are incorporated: conservative amino acid substitutions are frequently incorporated and up to 5 residue positions may be varied to incorporate non-conservative amino acid substitutions as compared to known naturally-occurring CDR sequences. Such CDR sequences can be used in primary library members (prior to first round screening) and/or can be used to spike *in vitro* shuffling reactions of selected library member sequences. Construction of such pools of defined and/or degenerate sequences will be readily accomplished by those of ordinary skill in the art.

The collection of synthetic CDR sequences comprises at least one member that is not known to be a naturally-occurring CDR sequence. It is within the discretion of the practitioner to include or not include a portion of random or pseudorandom sequence corresponding to N region addition in the heavy chain CDR; the N region sequence ranges from 1 nucleotide to about 4 nucleotides occurring at V-D and D-J junctions. A collection of synthetic heavy chain CDR sequences comprises at least about 100 unique CDR sequences, typically at least about 1,000 unique CDR sequences, preferably at least about 10,000 unique CDR sequences, frequently more than 50,000 unique CDR sequences; however, usually not more than about 1×10^6 unique CDR sequences are included in the collection, although occasionally 1×10^7 to 1×10^8 unique CDR sequences are present, especially if conservative amino acid substitutions are permitted at positions where the conservative amino acid substituent is not present or is rare (*i.e.*, less than 0.1 percent) in that position in naturally-occurring human CDRS. In general, the number of unique CDR sequences included in a library should not exceed the expected number of primary transformants in the library by more than a factor of 10. Such single-chain antibodies generally bind of about at least 1×10^{-6} , preferably with an affinity of about at least 5×10^{-7} (superscript 7) M-1, more preferably with an affinity of at least 1×10^{-8} (superscript 8) M-1 to 1×10^{-9} (superscript 9) M-1 or more, sometimes up to 1×10^{-10} (superscript 10) M-1 or more. Frequently, the predetermined antigen is a human protein, such as for example a human cell surface antigen (e. g., CD4, CD8, IL-2 receptor, EGF receptor, PDGF

receptor), other human biological macromolecule (*e.g.*, thrombomodulin, protein C, carbohydrate antigen, sialyl Lewis antigen, Lselectin), or nonhuman disease associated macromolecule (*e.g.*, bacterial LPS, virion capsid protein or envelope glycoprotein) and the like.

8.16.5.8.10 Expression systems

High affinity single-chain antibodies of the desired specificity can be engineered and expressed in a variety of systems. For example, scfv have been produced in plants (Firek *et al.* (1993) Plant Mol. Biol. 23: 861) and can be readily made in prokaryotic systems (Owens RJ and Young RJ (1994) J. Immunol. Meth. 168: 149; Johnson S and Bird RE (1991) Methods Enzymol 203: 88). Furthermore, the single-chain antibodies can be used as a basis for constructing whole antibodies or various fragments thereof (Kettleborough *et al.* (1994) Eur. J. Immunol. 24: 952). The variable region encoding sequence may be isolated (*e.g.*, by PCR amplification or subcloning) and spliced to a sequence encoding a desired human constant region to encode a human sequence antibody more suitable for human therapeutic uses where immunogenicity is preferably minimized. The polynucleotide(s) having the resultant fully human encoding sequence(s) can be expressed in a host cell (*e.g.*, from an expression vector in a mammalian cell) and purified for pharmaceutical formulation.

The DNA expression constructs will typically include an expression control DNA sequence operably linked to the coding sequences, including naturally-associated or heterologous promoter regions. Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and the collection and purification of the mutant "engineered" antibodies.

As stated previously, the DNA sequences will be expressed in hosts after the sequences have been operably linked to an expression control sequence (*i.e.*, positioned to ensure the transcription and translation of the structural gene). These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection

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In addition to eukaryotic microorganisms such as yeast, mammalian tissue cell culture may also be used to produce the polypeptides of the present invention (see, Winnacker, "From Genes to Clones," VCH Publishers, *N.i., N.Y.* (1987), which is incorporated herein by reference). Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact immunoglobulins have been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa cells, and myeloma cell lines, but preferably transformed Bcells or hybridomas. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen *et al.* (1986) Immunol. Rev. 89: 49), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, cytomegalovirus, SV40, Adenovirus, Bovine Papilloma Virus, and the like.

Eukaryotic DNA transcription can be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting sequences of between 10 to 300 bp that increase transcription by a promoter. Enhancers can effectively increase transcription when either 51 or 31 to the transcription unit. They are also effective if located within an intron or within the coding sequence itself. Typically, viral enhancers are used, including SV40 enhancers, cytomegalovirus enhancers, polyoma enhancers, and adenovirus enhancers. Enhancer sequences from mammalian systems are also commonly used, such as the mouse immunoglobulin heavy chain enhancer.

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identified by their ability to grow on non-supplemented media. Examples of prokaryotic drug resistance genes useful as markers include genes conferring resistance to G418, mycophenolic acid and hygromycin.

The vectors containing the DNA segments of interest can be transferred into the host cell by well-known methods, depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment, lipofection, or electroporation may be used for other cellular hosts. Other methods used to transform mammalian cells include the use of Polybrene, protoplast fusion, liposomes, electroporation, and micro-injection (see, generally, Sambrook *et al.*, *supra*).

Once expressed, the antibodies, individual mutated immunoglobulin chains, mutated antibody fragments, and other immunoglobulin polypeptides of the invention can be purified according to standard procedures of the art, including ammonium sulfate precipitation, fraction column chromatography, gel electrophoresis and the like (see, generally, Scopes, R., Protein Purification, Springer-Verlag, N.Y. (1982)). once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically or in developing and performing assay procedures, immunofluorescent stainings, and the like (see, generally, Immunological Methods, Vols. I and II, Eds. Lefkovits and Pernis, Academic Press, New York, N.Y. (1979 and 1981)).

The antibodies generated by the method of the present invention can be used for diagnosis and therapy. By way of illustration and not limitation, they can be used to treat cancer, autoimmune diseases, or viral infections. For treatment of cancer, the antibodies will typically bind to an antigen expressed preferentially on cancer cells, such as erbB-2, CEA, CD33, and many other antigens and binding members well known to those skilled in the art.

8.16.5.9 Yeast Two-Hybrid Screening Assays

Shuffling can also be used to recombinatorially diversify a pool of selected library members obtained by screening a two-hybrid screening system to identify library members

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An approach to identifying polypeptide sequences which bind to a predetermined polypeptide sequence has been to use a so-called "two-hybrid" system wherein the predetermined polypeptide sequence is present in a fusion protein (Chien *et al.* (1991) Proc. Natl. Acad. Sci. (USA) 88: 9578). This approach identifies protein-protein interactions *in vivo* through reconstitution of a transcriptional activator (Fields S and Song 0 (1989) Nature 340: 245), the yeast Gal4 transcription protein. Typically, the method is based on the properties of the yeast Gal4 protein, which consists of separable domains responsible for DNA-binding and transcriptional activation. Polynucleotides encoding two hybrid proteins, one consisting of the yeast Gal4 DNA-binding domain fused to a polypeptide sequence of a known protein and the other consisting of the Gal4 activation domain fused to a polypeptide sequence of a second protein, are constructed and introduced into a yeast host cell. Intermolecular binding between the two fusion proteins reconstitutes the Gal4 DNA-binding domain with the Gal4 activation domain, which leads to the transcriptional activation of a reporter gene (*e.g.*, *lacZ*, *HIS3*) which is operably linked to a Gal4 binding site. Typically, the two-hybrid method is used to identify novel polypeptide sequences which interact with a known protein (Silver SC and Hunt SW (1993) Mol. Biol. Rep. 17: 155; Durfee *et al.* (1993) Genes Devel. 7: 555; Yang *et al.* (1992) Science 257: 680; Luban *et al.* (1993) Cell 73: 1067; Hardy *et al.* (1992) Genes Devel. 6: 801; Bartel *et al.* (1993) Biotechniques 14: 920; and Vojtek *et al.* (1993) Cell 74: 205). However, variations of the two-hybrid method have been used to identify mutations of a known protein that affect its binding to a second known protein (Li B and Fields S (1993) FASEB J. 7: 957; Lalo *et al.* (1993) Proc. Natl. Acad. Sci. (USA) 90: 5524; Jackson *et al.* (1993) Mol. Cell. Biol. 13: 2899; and Madura *et al.* (1993) J. Biol. Chem. 268: 12046). Two-hybrid systems have also been used to identify interacting structural domains of two known proteins (Bardwell *et al.* (1993) med. Microbial. 8: 1177; Chakrabarty *et al.* (1992) J. Biol. Chem. 267: 17498; Staudinger *et al.* (1993) J. Biol. Chem. 268: 4608; and Milne GT. and Weaver DT (1993) Genes Devel. 7: 1755) or domains responsible for oligomerization of a single protein (Iwabuchi *et al.* (1993) Oncogene 8: 1693; Bogerd *et al.* (1993) J. Virol. 67: 5030). Variations of two-hybrid

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systems have been used to study the *in vivo* activity of a proteolytic enzyme (Dasmahapatra *et al.* (1992) Proc. Natl. Acad. Sci. (USA) 89: 4159). Alternatively, an E. coli/BCCP interactive screening system (Germino *et al.* (1993) Proc. Natl. Acad. Sci. (U.S.A.) 90: 933; Guarente L (1993) Proc. Natl. Acad. Sci. (U.S.A.) 90: 1639) can be used to identify interacting protein sequences (*i.e.*, protein sequences which heterodimerize or form higher order heteromultimers). Sequences selected by a two-hybrid system can be pooled and shuffled and introduced into a two-hybrid system for one or more subsequent rounds of screening to identify polypeptide sequences which bind to the hybrid containing the predetermined binding sequence. The sequences thus identified can be compared to identify consensus sequence(s) and consensus sequence kernels.

In general, standard techniques of recombination DNA technology are described in various publications, *e.g.* Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory; Ausubel *et al.*, 1987, Current Protocols in Molecular Biology, vols. 1 and 2 and supplements, and Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, CA, each of which is incorporated herein in their entirety by reference. Polynucleotide modifying enzymes were used according to the manufacturers recommendations. Oligonucleotides were synthesized on an Applied Biosystems Inc. Model 394 DNA synthesizer using ABI chemicals. If desired, PCR amplimers for amplifying a predetermined DNA sequence may be selected at the discretion of the practitioner.

8.16.5.9.1 Formation of Dimers

One microgram samples of template DNA are obtained and treated with U.V. light to cause the formation of dimers, including TT dimers, particularly purine dimers. U.V. exposure is limited so that only a few photoproducts are generated per gene on the template DNA sample. Multiple samples are treated with U.V. light for varying periods of



8.16.5.9.4 Generating Libraries Suitable for Affinity Interaction Screening or Phenotypic Screening

In one embodiment, the present invention provides a method for generating libraries of displayed polypeptides or displayed antibodies suitable for affinity interaction screening or phenotypic screening. The method comprises (1) obtaining a first plurality of selected library members comprising a displayed polypeptide or displayed antibody and an associated polynucleotide encoding said displayed polypeptide or displayed antibody, and obtaining said associated polynucleotides or copies thereof wherein said associated polynucleotides comprise a region of substantially identical sequences, optimally introducing mutations into said polynucleotides or copies, (2) pooling the polynucleotides or copies, (3) producing smaller or shorter polynucleotides by interrupting a random or particularized priming and synthesis process or an amplification process, and (4) performing amplification, preferably PCR amplification, and optionally mutagenesis to homologously recombine the newly synthesized polynucleotides.

8.16.5.9.5 Producing Hybrid Polynucleotides Which Express a Useful Hybrid Polypeptide

It is a particularly preferred object of the invention to provide a process for producing hybrid polynucleotides which express a useful hybrid polypeptide by a series of steps comprising:

- (a) producing polynucleotides by interrupting a polynucleotide amplification or synthesis process with a means for blocking or interrupting the amplification or synthesis process and thus providing a plurality of smaller or shorter polynucleotides due to the replication of the polynucleotide being in various stages of completion;
- (b) adding to the resultant population of single- or double-stranded polynucleotides one or more single- or double-stranded oligonucleotides, wherein said

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added oligonucleotides comprise an area of identity in an area of heterology to one or more of the single- or double-stranded polynucleotides of the population;

(c) denaturing the resulting single- or double-stranded oligonucleotides to produce a mixture of single-stranded polynucleotides, optionally separating the shorter or smaller polynucleotides into pools of polynucleotides having various lengths and further optionally subjecting said polynucleotides to a PCR procedure to amplify one or more oligonucleotides comprised by at least one of said polynucleotide pools;

(d) incubating a plurality of said polynucleotides or at least one pool of said polynucleotides with a polymerase under conditions which result in annealing of said single-stranded polynucleotides at regions of identity between the single-stranded polynucleotides and thus forming of a mutagenized double-stranded polynucleotide chain;

(e) optionally repeating steps (c) and (d);

(f) expressing at least one hybrid polypeptide from said polynucleotide chain, or chains; and

(g) screening said at least one hybrid polypeptide for a useful activity.

In a preferred aspect of the invention, the means for blocking or interrupting the amplification or synthesis process is by utilization of uv light, DNA adducts, DNA binding proteins.

In one embodiment of the invention, the DNA adducts, or polynucleotides comprising the DNA adducts, are removed from the polynucleotides or polynucleotide pool, such as by a process including heating the solution comprising the DNA fragments prior to further processing.

Having thus disclosed exemplary embodiments of the present invention, it should be noted by those skilled in the art that the disclosures are exemplary only and that various other alternatives, adaptations and modifications may be made within the scope of the

present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following examples are to be considered illustrative and thus are not limiting of the remainder of the disclosure in any way whatsoever.

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Unless otherwise indicated, all references cited herein (supra and infra) are incorporated by reference in their entirety.

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$\{f_{\alpha}^{(1)}\}$ and $\{f_{\alpha}^{(2)}\}$ are the first and second order Fourier coefficients of f and g respectively. The first order coefficients are given by

$$f_{\alpha}^{(1)} = \frac{1}{2\pi} \int_0^{2\pi} f(\theta) e^{-i\alpha\theta} d\theta$$
 and the second order coefficients are given by

$$f_{\alpha}^{(2)} = \frac{1}{2\pi} \int_0^{2\pi} f(\theta) e^{-i\alpha\theta} d\theta$$
 where α is a positive integer. The first order coefficients are the Fourier coefficients of f and the second order coefficients are the Fourier coefficients of g . The first order coefficients are the Fourier coefficients of f and the second order coefficients are the Fourier coefficients of g .

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